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THE ESTIMATION OF LATENT INFECTION IN ORANGES*

By D. B. ADAM,† JEAN McNEIL, B. M. HANSON-MERZ, D. F. MCCARTHY, and
JOAN STOKES

[Manuscript received February 18, 1949]

(Plate 1)

Summary

The main objective of work described in this paper was to find out what differences occurred in the degree of latent infection of Washington Navel oranges grown under different circumstances and treated in various ways. Observations were also made of fungi occurring as latent infections and on the conditions for, and mode of, infection by *Colletotrichum gloeosporoides*, the principal form concerned.

Estimates of the degree of infection were based on results obtained by plating pieces taken from the rind of apparently sound oranges. The methods used are described. *Colletotrichum gloeosporoides* was easily the most common and widespread form found. *Alternaria* spp. also occurred generally but much less frequently. *Phoma citricarpa* and *Septoria citricola* were characteristic of oranges from certain places.

With respect to infection by *Colletotrichum* it was found that significant differences may occur between different parts of the orange, between oranges from different trees in the same grove, and between oranges from different districts. Significant differences may also occur between oranges taken in successive years from the same trees.

The question of the time that infection by *Colletotrichum* occurs was examined under natural conditions and under conditions where the inoculation was controlled. With natural conditions in South Australia, the first significant increase in the degree of infection occurred in April or May and was often followed by further significant increases during the winter months. In experiments where inoculation was controlled, infections occurred as the result of inoculating oranges in January; however, they were more frequent with inoculations in March and later months.

Infections with *Septoria citricola* were also established by using appropriate spore suspensions. Here, inoculations made in April were the most successful. Observations on the time required by *Colletotrichum* to establish itself within the rind tissues revealed that in a humid atmosphere at 25°C. relatively little infection occurs in less than 24 hours, a fact which no doubt accounts at least in part for the freedom from infection of South Australian oranges during the hot dry weather that is normal for the period October–April/May.

* The work described in this paper was commenced in 1939. The four junior authors were each associated with it in turn for about twelve months.

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Applications of Bordeaux mixture reduced latent infection to a degree that varied according to the times of application. The effect of curing or "sweating" oranges was not consistent from season to season. During two seasons a relatively small but significant reduction was effected but this did not occur in the third season.

Storage of oranges at 4.5°C. for six weeks had no significant effect on their degree of infection.

The microscopic features of the infection process are discussed briefly and agree substantially with those described for tropical fruits by Simmonds (1941).

I. INTRODUCTION

Interest in the circumstances of infection of citrus and other fruits by *Colletotrichum gloeosporoides* and by other fungi has revived in recent years. This is evident from contributions by Bates (1936) from Southern Rhodesia, Baker and Wardlaw (1937) and Baker, Crowdy, and McKee (1940) from Trinidad who considered the problem in citrus fruits particularly. Simmonds (1941) extended observations to the similar phenomenon in various tropical fruits, especially the banana.

Shear and Wood (1913) were perhaps the first investigators to draw attention to the fact that *Glomerella cingulata* (Stonem.) Sp. and von S. and homologues referable to the form genera *Colletotrichum* and *Gloeosporium*, was able to remain in a dormant or quiescent condition in the leaves, stems, and fruits of a wide range of host plants. In drawing attention to this behaviour as a feature of infection by *Glomerella* they suggested that the quiescent state remains "until the host becomes weakened in some way or until specially favourable conditions for the fungus occur." They also pointed out that "in many cases the fungus never develops until the infected part of the host dies."

Our interest in the latent infection of oranges arises from the fact that after a period of cool storage citrus fruits, especially grape fruit and Washington Navel oranges, develop a disorder of the pericarp commonly described as "storage spot" or "pitting." Opinions concerning the etiology of this disease vary. According to one view, the spotting is due to a disturbed metabolism in orange pericarp occasioned by the circumstances of storage, whilst another view ascribes some role to the presence of pathogenic organisms in the tissues of the pericarp. In the present account, however, we do not consider these differences of viewpoint and are simply concerned with the question of the extent to which latent infection by potentially pathogenic fungi occurs.

Latent infections in fruits may be studied by either one of two methods. They may be studied directly, in microscopic preparations, as was done by Simmonds (1941), or less directly from results obtained by plating treated pieces of apparently healthy orange rind tissues on media suitable for the growth of the organism sought. This latter method, used by Baker and Wardlaw (1937) and others, is advantageous in that it is possible to examine numerous pieces of tissue either from a single orange or from several oranges which may have come from populations with different histories. The method therefore provides a means for considering data quantitatively.

Most of the results described below arose from the application of methods involving the plating of pieces of orange rind, but some consideration was also given to the development of infection under controlled conditions of inoculation and to the microscopic details of infection.

II. EXPERIMENTAL PROCEDURE

(a) *Estimating the Degree of Latent Infection*

(i) *Isolation Methods.*—The method of disinfecting the surfaces of apparently healthy oranges from which isolations were to be made was essentially that used by Baker and Wardlaw (1937) with the difference that, Agral 2 (a sulphonated organic wetting agent) was incorporated with the disinfectants used. This was done because preliminary trials revealed that the addition of Agral 2 served to eliminate practically all the few miscellaneous fungi which, it was suspected, were present as surface contaminants rather than as latent infections.

The following standard procedure was adopted. The oranges to be disinfected were:

- (1) Immersed for ten minutes in a saturated solution of borax + 0.45% Agral 2 at 45°C.
- (2) Washed in two changes of sterile water.
- (3) Immersed for ten minutes in 0.1% mercuric chloride + 0.45% Agral 2 at room temperature.
- (4) Washed in two changes of sterile water.

Subsequent to surface disinfection small round pieces of rind tissue were removed with a sterile cork borer (5 mm.) and planted on malt agar plates (25 g. malt extract, 22 g. agar to 1 l. water).

The number of pieces taken from each orange varied from five, in the earlier stages, to fifteen later on. The pieces were selected at random, either from the orange as a whole or from a particular region such as the "stem end," defined as a region extending for about two inches from the stem connexion. With the exception of some oranges sampled after cool storage the oranges were selected for freedom from visible defects. Special precautions were taken to minimize the chances of contamination of plates from external sources.

The value of any isolation method in establishing the existence of latent infection turns on the question whether fungi developing on the isolation plate have developed from hyphae within the tissues or from spores or other fungal structures on the surface of the orange. Early in the investigation the following experiment was undertaken to throw more light on this point. Twenty-four fully grown oranges were selected from a sample which, when examined by the isolation method described, showed no evidence of latent infection. Half of the surface of each of these oranges was inoculated with a spore suspension of *Colletotrichum gloeosporoides* applied with an atomizer and allowed to dry immediately. Three days after inoculation twelve of the oranges were

disinfected by the standard procedure and the remainder left to be used as controls. Six pieces, selected at random, were plated from each half of the twenty-four oranges. Forty-seven pieces of the 72 pieces, i.e. 65 per cent. of those plated from the inoculated halves of the non-disinfected oranges, yielded *Colletotrichum* on culturing but none of the pieces from either half of the surface disinfected oranges or from the uninoculated halves of the non-disinfected oranges yielded any growth of fungus. Obviously the disinfection process employed completely inhibited growth from surface borne spores of *Colletotrichum*. Further observations on infection processes are reserved for discussion later.

(ii) *Statistical Considerations.*—In the sequel it will be seen that populations of oranges, whether they be from different trees, different districts, or be subject to different treatments, vary in the number of pieces per orange yielding a fungous growth, and an estimate of the significance of these differences was desired. The matter was examined in a preliminary experiment. One

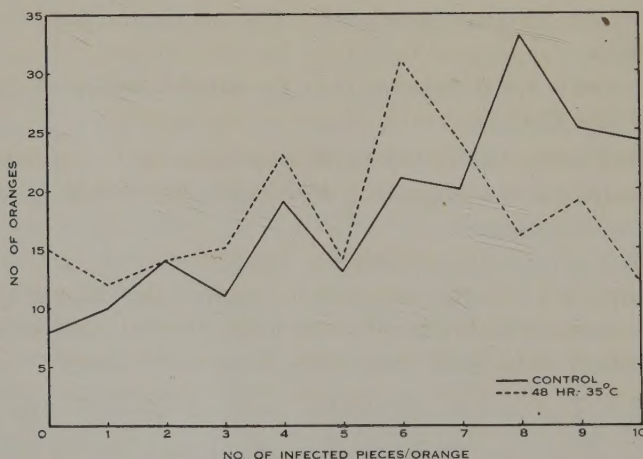


Fig. 1.—The distribution of two populations of oranges in terms of the number of infected pieces (out of ten) per orange.

hundred and thirty-four oranges were picked from each of three trees, divided equally into two groups and then bulked to give lots of 201 oranges. One of these lots was cured or “sweated” by keeping the fruit in a thermostatically controlled cabinet at 35°C. for two days. Each lot was examined for latent infection as soon as possible after picking or after the curing treatment. Ten pieces were plated from each orange, five at random positions from the “stem end” and five from random positions on the remainder of the orange. When examined subsequently, practically the only fungal growth present was that of *Colletotrichum*. The distributions of the two orange populations in terms of numbers of pieces yielding *Colletotrichum* are shown in Figure 1.

Consideration of the evidence regarding the effect of the sweating treatment is reserved till later when other evidence concerning such treatments is given.

On the statistical problem which this experiment was designed to elucidate, E. A. Cornish* kindly offered the following comment. "The method of determining the latent infection present provides a discrete variable of finite range. For a variable of this nature the binomial distribution might be considered to provide a suitable basis for testing the significance of treatment difference but it cannot be used because every piece of rind of specified size has not an equal and independent chance of becoming infected. In seeking an alternative method of analysis, we may note first that the score assigned to any orange, viz. the number of pieces out of ten plated, can be taken as a sampling estimate of the amount or degree of infection on that orange. In the second place any correlation which may exist between observations on the same orange does not invalidate the score assigned to that orange as a variable for estimating the degree of infection or for use in an analysis.

"The discontinuity of the variable will to some extent affect the analysis based on the normal distribution, but even with a range as small as that used this effect will not be great. It might be reduced if the number was increased to say fifteen" (a procedure adopted in later work). "The skewness of the distribution probably influences tests of significance more than the discontinuity of the variable but provided that counts are distributed over the greater part of the range as they are in this case they will not affect the result appreciably."

In cases then where results were similar in character to the above it has been assumed for purposes of analysis that distribution was normal.

(b) Inoculation Experiments

It was suspected that the extent of infection with *Colletotrichum* or other fungi which can establish latent infection under natural conditions was related to the incidence of rainfall. If trees could be protected from rain the infection of oranges would be minimized. To accomplish this a glass roof was erected over four fully grown Washington Navel orange trees. The sides of this structure were equipped with roller blinds which could be let down when rain occurred. For the first two years this structure was located in a private orange grove at Mypolonga, South Australia, and for the third season in the Government orchard at Fullarton. The structure was generally effective for the purposes intended but the occurrence of light, driving rain entering from the sides could not always be anticipated. The trees grew well, bore normal crops of oranges, and did not appear to be affected in any way by the covering. They were watered by furrow irrigation.

Inoculation was effected by atomizing spore suspensions on to the uninjured surface of unblemished oranges, and, to overcome possible variations in the virulence of different strains, mixtures of several single spore isolates were used. The maintenance of moist conditions in the infection court for a sufficient period for infection to occur was difficult with inoculations effected during hot dry

* Formerly at the Waite Agricultural Research Institute, now Officer-in-Charge, Section of Mathematical Statistics, C.S.I.R.

weather. To achieve this in later experiments, the following procedure was adopted. After applying the spore suspension, the droplets were first allowed to dry and then the oranges were covered with a thin pad of cotton wool wetted with sterile water and fairly well wrung out. This was then enclosed in closely fitted tinfoil and finally in paraffin-treated paper bags tied securely around the stem. Evidence of infection was secured by plating pieces of tissue from inoculated oranges by methods previously described.

(c) *Study of Microscopic Features of Infection*

Oranges protected from natural infection were picked during May and June and their surfaces disinfected by immersion in 70 per cent. alcohol. Defined areas were inoculated with a heavy spore suspension of *Colletotrichum* and maintained for periods of 12-143 hours in a moist atmosphere at 25°C., after which hand or microtome sections were cut. For embedding, pieces of the rind were fixed in formalin-aceto-alcohol, while for dehydration tertiary butyl alcohol was used (Johansen 1940). The cotton blue-lactophenol combination used by Simmonds (1941) was not found very satisfactory for tracing hyphae below the cuticle. Better results were obtained with the following schedule:

- (1) Stain with 0.1% thionin in 5% aqueous phenol for one hour.
- (2) Counterstain with orange G in absolute alcohol or clove oil for 30-60 seconds.
- (3) Clear with methyl salicylate; mount in Canada balsam.

The use of thionin in this way (Johansen 1940) stains the delicate intercellular hyphae blue but the material in which they are embedded appears reddish owing to the metachromatic property of thionin. Care with the period of counterstaining was the principal precaution required in the use of this procedure.

III. RESULTS

The main part of the investigation was concerned with estimating the latent infection of oranges growing naturally in different districts or subjected to different treatments and the data obtained can be considered most conveniently in the series of subsections which follow.

(a) *Fungi Present as Latent Infections*

Colletotrichum gloeosporoides was easily the most common species isolated from the rind of apparently sound Washington Navel oranges grown in districts mentioned later. Its prevalence may be illustrated by reference to an experiment where freshly picked oranges from six districts, three in New South Wales, two in South Australia, and one from Victoria were sampled. Six hundred and sixty-five oranges, drawn almost equally from these districts, were sampled by plating twelve pieces from each orange. Three thousand and twenty-two of the pieces (38.6 per cent.) plated yielded some fungal growth and of these

89.4 per cent. were identified as *Colletotrichum gloeosporoides*. Furthermore, under circumstances where the number of infected pieces was high, a matter dealt with later, the proportion of isolates identifiable as *Colletotrichum gloeosporoides* was also high. The point may be illustrated by reference to the sampling of oranges from Griffith, New South Wales. From 64 per cent. of the pieces plated fungi grew and of these 97 per cent. were identified as *Colletotrichum*.

Of other fungi developing on the isolation plates, *Phoma citricarpa*, *Septoria citricola*, and *Alternaria* spp. were most noteworthy. *Phoma* and *Septoria* only occurred on a small proportion of the pieces plated, e.g. 1.8 and 2.3 per cent., though the number of oranges (18-21 per cent.) from which such pieces came was quite significant in some consignments. The point of special interest about these fungi was their restricted distribution. *Phoma citricarpa* occurred only in consignments from the Gosford district whilst *Septoria* was found only in consignments from the Murrumbidgee Irrigation Area and from South Australia. Both these findings agree with what is known about the distribution of "spot" diseases caused by these fungi and it is evident that like *Colletotrichum* they may occur as latent infections (Kiely 1948).

The fungi identified as *Alternaria* spp. were very variable in their growth characteristics; some agreed quite closely with descriptions of *A. citri* but specific identification was not generally pursued. *Alternaria* like *Colletotrichum* was found (though usually in much lower numbers) in oranges from all districts. Occasionally, however, where infection with *Colletotrichum* was low, as was generally the case with oranges from the Upper Murray Irrigation Areas of South Australia, the proportion of isolates identifiable as *Alternaria* might be quite high; in one sampling of 150 oranges from Waikerie, South Australia, 5.5 per cent. of the pieces yielded *Colletotrichum* and 6.4 per cent. *Alternaria*.

(b) *Variations in Latent Infection in Oranges from Different Districts, in Oranges from Different Trees in One District, and in Different Parts of a Single Orange*

Samples of five sound oranges taken at random from each of twelve healthy trees growing in eight districts were obtained in 1939 and examined by methods previously described. Twelve pieces were plated from each orange, six from the "stem end" and six from the remainder of the orange. Twelve hundred pieces were therefore examined from each grove. The results showed that significant variations occurred between oranges from different districts, from different trees in each grove, and from different parts of the orange. The degree of infection found in oranges from different districts is given in Table 1 where, for the sake of facilitating comparison with data in other tables, the number of infected pieces is expressed as a percentage of all the pieces plated.

The higher degree of latent infection in the oranges from New South Wales is quite evident. It will be noted that these oranges were the first lots picked but this fact is unlikely to account for the differences since later data show that if there is a significant change in the degree of latent infection it increases in later pickings.

Differences in infection between oranges from different trees in one grove and between different parts of a single orange were evident in oranges from all districts but most marked in oranges where latent infection was generally high.

TABLE 1
DEGREE OF INFECTION IN ORANGES FROM DIFFERENT DISTRICTS 1939

District	Picking Date	Pieces with <i>Colletotrichum</i> (%)
Griffith, N.S.W.	April 24	62
Somersby, Gosford, N.S.W.	May 8	54
Mangrove Mountain, Gosford, N.S.W.	May 8	49
Mildura, Vic.	May 29	Trace
Berri, S.A.	June 18	3
Waikerie, S.A.	June 21	1
Mypolonga, S.A.	July 17	3
Torrens Valley, S.A.	July 29	14

The differences between oranges from different trees may be illustrated by the data with oranges from Mangrove Mountain, Gosford. In this case where the mean infection of all oranges was 49 per cent. the degree of infection of oranges from tree to tree varied from 8.92 ± 8.1 per cent. Actually, on the basis of the latent infection of the oranges they bore, the trees fell into three groups of almost equal numbers with infections of 89 ± 6.8 , 36 ± 6.8 , and 19 ± 7.4 per cent. respectively. It might have been expected that differences of this order would be reflected in the health and vigour of these trees since small dead twigs in unthrifty trees often carry the fructifications of *Colletotrichum* but neither in these trees nor in others where similar differences occurred could the trees be

TABLE 2
MEAN PERCENTAGE LATENT INFECTION IN DIFFERENT PARTS OF ORANGES FROM
NEW SOUTH WALES 1939

District	Infected Pieces		Standard Error (\pm)
	"Stem End" (%)	Remainder of Orange (%)	
Griffith	84	41	2.5
Somersby	68	39	2.4
Mangrove Mountain	59	40	2.6

differentiated on their appearance in the orchard. Actually, this was to be expected since in the first instance the trees used had been selected for uniformity and thriftiness.

Differences in infection in different parts of the orange are again most evident in the more heavily infected oranges and may be illustrated by reference to the results obtained with the oranges from New South Wales (Table 2).

The "stem end" is here defined as an area with a radius of about two inches from the "button" or stem connexion. In some preliminary work, samples were taken from the "stem end," the navel end, and other parts of the orange which might be defined but the results suggested that the most useful distinction was that referred to in Table 2. It was used throughout the investigations and provided results consistent with those quoted above.

(c) *When Does Infection Occur?*

(i) *Under Natural Conditions.*—Estimates of latent infection at intervals subsequent to the time that the fruit "sets" were obtained for oranges from two districts over two years. For each occasion the estimate was obtained by sampling 30 oranges drawn in equal numbers from the same three trees. In the first season ten pieces and in the second season fifteen pieces taken at random from the orange were examined in the usual manner. The results of these examinations are given in Table 3.

TABLE 3
MEAN PERCENTAGE LATENT INFECTION IN ORANGES SAMPLED AT INTERVALS FROM
"FRUIT SETTING" TO MATURITY

District	Month of Sampling								
	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.
Athelstone 1940-41	0.7*	1.0*	2	3	37†	25	38	63	70
Athelstone 1942	—	0.7*	0	0.7	0.5	32	52	70	—
Waikerie 1940-41	0.7*	0.3*	7	8	6	26	29	25	25
Waikerie 1942	—	0.7*	0.2*	0.2	1.3	52‡	55	62	—

* These figures refer to the presence of *Alternaria* spp.; there were no colonies of *Colletotrichum*.

† Figures in bold type are significantly greater than those for preceding dates of sampling.

‡ This sample was actually taken early in June, six weeks subsequent to the previous sample and a fortnight prior to the next one.

From Table 3 it can be seen that for these two South Australian districts the first significant increase of infection occurred in April or May. The end of April and May are the times when the autumn rains, characteristic of the South Australian climate, usually commence but no clear correlation between rainfall and degree of infection can be established with the limited data available. Rainfall, however, may not be the only important element in promoting infection. In 1941 abundant rains fell in South Australia at the end of January and again at the beginning of March but neither of these occasions led to significant changes in the infection of oranges sampled shortly afterwards. Air temperature, humidity, and wind in their effect on the persistence of moisture on the surface of oranges might all be important but the limited information available does not warrant further pursuit of the subject here.

Two further points about the data in Table 3 may be noted. At Athelstone, close to Adelaide where rainfall is considerably greater than at Waikerie, the degree of infection increased significantly later in both seasons and by July had

reached a fairly uniform and high figure. At Waikerie no second significant increase occurred but there was a significant difference between infection in the two seasons. Evidence confirmatory of this variation from season to season was obtained from a grove at Mypolonga where, over a four year period, in oranges sampled at closely corresponding dates, latent infections of 43.2, 3.3, 18.6, and 43.9 per cent. respectively, were found in successive years.

(ii) *Under Controlled Conditions.*—These experiments were conducted over a three year period with oranges grown on trees under the cover described earlier. Each season oranges on two of the trees were inoculated with a spore suspension of *Colletotrichum* while those on the other two trees were inoculated with *Septoria*. Fifteen to twenty oranges on each tree were inoculated on each occasion but for various reasons not all these were available on the date when all oranges were picked for examination. Controls in the experiment were provided by oranges borne on the same trees as those inoculated; they differed only in that they were not atomized with the appropriate spore suspension.

TABLE 4
INFECTION OF ORANGES INOCULATED ON VARIOUS DATES WITH *COLLETOTRICHUM*,
MYPOLONGA 1941

Date of Inoculation	Oranges Examined (No.)	Pieces Yielding Growth (%)	Oranges Infected (No.)
March 4	19	45.0	17
April 8	20	55.0	19
July 22	28	43.0	26
Uninoculated Group A*	39	10.3	10
Uninoculated Group B	146	1.1	11

* This group was taken from the two trees on which other oranges were inoculated with *Colletotrichum*. The two trees were both on the western side of the enclosure and thus more exposed to risks from light, driving rain entering from the side. Group B oranges were taken from trees on which other oranges had been inoculated with *Septoria*. In their position they were also less exposed to the risks of wetting by rain.

In 1939-40, a total of 116 oranges inoculated in groups on seven occasions extending from November 7 to May 25 were examined in July for evidence of infection but the highest percentage of infected pieces secured, the result of inoculation on February 22, was only 6.2 per cent. The position with *Septoria* where 98 oranges in all were examined, was similar; only 1.2 per cent. of infected pieces were obtained as the result of inoculations on the same date. Of 53 oranges serving as controls in this experiment, none yielded any evidence of infection with either fungus.

In the following season more care was taken to ensure that adequate moisture on the orange surface was maintained during the period infection was likely to occur. These steps have been described and apparently they contributed to the more satisfactory results for inoculation with *Colletotrichum* shown in Table 4.

With *Septoria*, infections arising from inoculation were again infrequent. Forty oranges were inoculated on each of the three occasions mentioned in Table 4 but only two of the oranges inoculated on March 4 and one inoculated on July 22 yielded evidence of infection. No isolations of *Septoria* were obtained from any of the oranges serving as controls.

For the experiments in 1942 the glass roof was moved to the Government orchard at Fullarton. The rainfall there was heavier and more frequent than at Myppolonga and the effective protection of trees from driving rain more difficult. As in previous years oranges on one pair of trees were inoculated with *Colletotrichum*; while those on another were inoculated with *Septoria*. Results obtained with these inoculations are given in Table 5.

TABLE 5
INFECTION OF ORANGES INOCULATED ON VARIOUS DATES WITH EITHER
COLLETOTRICHUM OR *SEPTORIA*, FULLARTON 1942

Date of Inoculation	<i>Colletotrichum</i>			<i>Septoria</i>		
	Oranges Examined (No.)	Pieces Yielding <i>Colletotrichum</i> (%)	Oranges Infected (No.)	Oranges Examined (No.)	Pieces Yielding <i>Septoria</i> (%)	Oranges Infected (No.)
January 20	15	10.9	10	17	0.7	1
April 24	17	84.4	17	17	24.6	14
June 29	16	61.3	15	17	3.7	5
Uninoculated "controls"	56	5.2	9	43	1.2	3

(iii) *Time Required to Establish Infection.*—The difficulty of maintaining a film of moisture around artificially inoculated oranges prompted the making of a small experiment to ascertain the period necessary to establish infection. Fifteen oranges grown on one of the protected trees were selected and examined for evidence of natural infection by plating five pieces from each orange. They were then inoculated with a heavy suspension of *Colletotrichum* spores, divided into three lots of five, and kept in large Koch dishes at 25°C. with ample moisture. After 24, 48, and 72 hours respectively, one of the lots was removed and the oranges examined by plating five pieces before, and five pieces after, surface disinfection. The results of this experiment are given in Table 6.

The oranges used in this experiment were practically free from infection prior to their inoculation since *Colletotrichum* grew from only one of a total of 75 pieces plated from the oranges at this stage of the experiment. The figures in the last column of Table 6 reveal the degree of infection obtained with each period of incubation. Infection after 72 hours' incubation was close to the maximum possible under the conditions of the experiment. It was significantly greater than that obtained with 48 hours' incubation which, in turn, was signifi-

cantly greater than that with oranges incubated for 24 hours. The data in the third column can be taken as a measure of the potency of the inoculum used. The high, uniform figures for each group were contributed partly by the number of infections and partly by viable spores or young fungal growths which had not yet established themselves as infections, i.e. had not yet grown beyond the inhibitory action of the disinfectants used. The contribution of these two elements to the situation can be seen most clearly with oranges incubated for 24 hours.

TABLE 6

NUMBER OF PIECES (OF A POSSIBLE 25) YIELDING *COLLETOTRICHUM* BEFORE AND AFTER INOCULATION; INOCULATED ORANGES INCUBATED AT 25°C.

Incubation Period (hr.)	Isolations of <i>Colletotrichum</i>		
	Before Inoculation (No.)	After Inoculation	
		Before Disinfection (No.)	After Disinfection (No.)
24	0	23	3
48	1	21	13
72	0	23	21

Here the difference in numbers before and after disinfection was highly significant. The result obtained before disinfection was no doubt contributed to by surface borne spores and appressoria since these could be observed in hand sections made from these oranges. That these structures scarcely contributed to the figures in the last column, those on which the degree of infection is based, was confirmed by a later experiment. In this case, oranges free from evidence of infection were inoculated and incubated for a 13 hour period at 25°C. Hand sections revealed that spore germination and appressorial formation had occurred freely but after surface disinfection no pieces yielded *Colletotrichum*.

It is evident that hyphae etc. produced from these spores had not yet passed beyond the inhibitory effect of the disinfectant, a fact which was interpreted to mean that, while spore germination had occurred after 13 hours, infections were not then established. This experiment also throws further light on the minimal period required for infection to occur at 25°C. It lies between 13 and 24 hours.

(d) Effects of Spray Treatments on Latent Infection

Spraying trials conducted by the Department of Agriculture afforded an opportunity to study the effects of spray treatments on the degree of latent infection.

In 1939-40 Bordeaux mixture (3:3:100) was applied either once or twice to blocks of five trees at Mypolonga during the months shown in Table 7. When

the fruit was mature at the end of August, 45 oranges were drawn from each block and sampled for infection in the usual way. The results are summarized in Table 7.

TABLE 7

RESULTS OF TRIAL AT MYPOLONGA 1939-40 SHOWING THE LATENT INFECTION OF ORANGES FROM TREES SPRAYED WITH BORDEAUX MIXTURE (3:3:100) AT VARIOUS TIMES

Months When Spray Applied	A Dec. only	B Dec. Jan.	C Dec. April	D Dec. May	E April only	F No Spray
Latent infection (%)	4.25	0.2	0.2	8.3	0.7	18.6

The degree of infection found, even in unsprayed oranges, was low and there is some doubt about the validity of the usual test for significance. An alternative test devised by Pitman (1937) was therefore used. By using this test we found that the following comparisons were significant at $P \leq 0.01$, viz.:

- (1) Less latent infection occurred in oranges from sprayed trees.
- (2) Sprays applied in December only or in December and May were less effective than the other applications employed.

It should also be noted that there was no significant difference between oranges receiving a single application in April and those which, in addition, had been sprayed in December.

In this and other experiments where deposits of spray occur on some oranges, it might be suggested that the presence of copper would tend to inhibit the growth of *Colletotrichum* if it were present as a latent infection. The point was examined but it is not necessary to give details, for no evidence of any such inhibitory action was found even in cases where the deposit was fresh and heavy.

TABLE 8

RESULTS OF SPRAYING TRIALS AT MYPOLONGA AND WAIKERIE 1941

District	Date Of Spray Application	Sampling Date	Mean Percentage Latent Infection		Standard Error (\pm)
			Sprayed	Unsprayed	
Mypolonga	February 21	June 28	18.5	43.9	3.93
Mypolonga	April 24	Sept. 5	24.4	46.9	3.60
Waikerie	February 27	June 20	3.1	17.5	2.0
Waikerie	March 27	July 25	7.3	40.3	3.3

In the following season, observations were extended to include a spray trial on trees at Waikerie as well as at Mypolonga. Comparisons were limited to two groups of trees; the first sprayed twice with Bordeaux mixture 3:3:100 on the dates shown in Table 8 while a second group was not sprayed. The oranges from both places were sampled twice; each sample consisted of 50 oranges taken in equal numbers from five trees of each group. The results are summarized in Table 8.

In all cases the effects of spraying on latent infection were significant but it will be noted that infection of oranges on the sprayed trees, especially those at Mypolonga, reached a comparatively high figure. The spray schedule employed was not efficient when circumstances favoured a high degree of infection. The results from the second sampling confirm those of the first and in their general increase accord with other observations. The significant increase in infection at the July picking of the unsprayed oranges from Waikerie should also be noted.

(e) *Effect of "Sweating" Treatments on the Latent Infection of Oranges*

Since storage trials revealed that "sweating" treatments applied to oranges prior to their cool storage may reduce the subsequent development of storage spot (Huelin 1942) we were interested in the question of the effects of such treatments on the degree of latent infection. Figure 1, referred to previously, deals with two groups of oranges which differ from one another only in that one group had been "sweated" at 35°C. for 48 hours prior to sampling. The mean infection determined for the untreated sample was 50.8 per cent., while that for the sweated oranges was 7.2 per cent. less — a difference which is significant.

TABLE 9

EFFECTS OF VARIOUS "SWEATING" TREATMENTS WITH ORANGES GROWN AT ATHELSTONE, PICKED JUNE 24, 1940

Sweating Treatments	Latent Infection (%)	Standard Error (±)
16 hr., 49°C.	44.6	3.75
48 hr., 40.5°C.	38.0	4.11
6 days, 32°C.	40.2	4.33
Untreated	65.3	3.75

In 1940 more comprehensive observations were made. They took into account a wider variety of "sweating" treatments and in sampling more attention was given to the various factors already considered which contribute to the variability of latent infection. The results of these observations are summarized in Table 9.

Each of the treatments referred to in Table 9 effected a significant reduction in latent infection when compared with that for the untreated oranges but there was no significant difference between the various treatments used.

Oranges from the same group of trees were examined again in the following season for the effects of sweating treatments. They were picked for treatment on three occasions: in May, June, and July. The degree of infection in untreated oranges was estimated at 28.9, 31.9, and 62.0 per cent. respectively but on no occasion did sweating treatments at 29.5°C. for 7 days or 32°C. for 2, 5, 8, and 11 days respectively, effect a significant reduction in latent infection. "Sweating" then was not consistent in its effects; on some occasions it reduced, on others it had no significant effect on, the degree of latent infection.

(f) *Does the Degree of Infection Increase if Oranges are Stored?*

Many observations have been made on the extent of latent infection in oranges before and after a period of cool storage and they all support the same conclusion, viz. that during a storage period there is no overall increase in the degree of infection. Only two cases supporting this view need be quoted. In the first case a sample of 66 oranges taken from a larger batch which had been sweated at 35°C. for two days was examined for latent infection in the usual way by plating ten pieces from each orange. The remaining oranges were then stored at 4.5°C. for a period of six weeks. On removal, 5 per cent. had developed storage spot. Sixty-six of these oranges were again examined by plating ten pieces taken from positions determined by a method of random selection which took no account of whether a lesion was, or was not, present at the point of sampling. The estimate of infection prior to storage was 45.1 ± 3.4 per cent., that subsequent to storage 44.1 ± 3.1 per cent.; an insignificant difference.



Fig. 2.—The appressoria of *Colletotrichum gloeosporoides*. $\times 1000$.

In the second case, 30 oranges were taken from a larger group of similar oranges and examined for latent infection, immediately after picking, by plating 15 pieces from each orange. The remainder of this group was stored at 4.5°C. for six weeks during which time a proportion developed "storage spot." Twenty of these spotted oranges were selected for examination and 15 pieces were plated from apparently healthy areas on them. In addition, one piece was plated from each lesion which meant that from 19-30 pieces were plated from each orange. We are not concerned here with differences between apparently healthy and lesioned tissues in the latter group of oranges but only with estimates for the whole orange so as to compare them with similar oranges prior to storage. The estimate prior to storage was 62.0 ± 4.7 per cent.; that after storage 56.0 ± 19.0 per cent.; again the difference is not significant.

(g) *Microscopic Features of the Latent Infection of Oranges*

A full account of the histological structures associated with the latent infection of oranges would vary but slightly from the excellent account of

Simmonds (1941) concerning similar structures associated with the latent infection of tropical fruits. In briefly describing the microscopic features then it is only necessary to stress such differences as appeared in our preparations.

Soon after germination the spores of *Colletotrichum* develop a characteristic organ—the appressorium (Fig. 2). This is brown in colour and borne at the end of a short germ tube. Appressoria vary greatly in shape, a feature which seems to be determined by the surface on which they develop and to which they are very closely applied. A distinct pore, seen best when viewed from above, occurs on the appressorial wall in contact with the host. Simmonds describes and illustrates a peg-like structure in this position but our observations suggest that it is a much broader structure than the term peg suggests. It seems better described as a thickening of the lip of the pore. It projected from the main body of the appressorium and, in many cases, was partly sunken in the cuticle. Its function in attachment seemed clear. We could see no evidence of the presence of mucilaginous substances which, it has been suggested, serve a similar function.

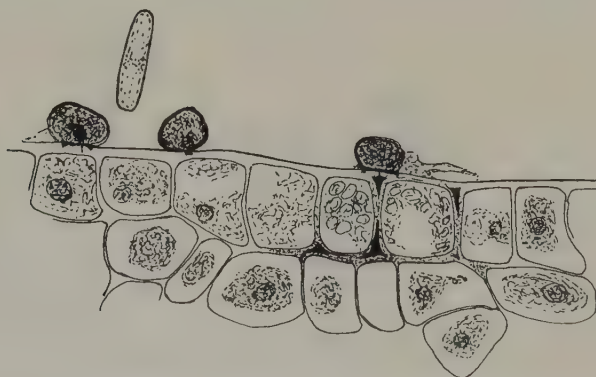


Fig. 3.—Penetration of pericarp tissues by hypha developing from an appressorium of *Colletotrichum*. $\times 800$.

The next stage in the infection process, the penetration of the cuticle, was observed only after prolonged search of sections stained with lactophenol-cotton blue. A fine infection thread, staining light blue and proceeding from the appressorial pore passes directly through the cuticle. After its passage the hypha, though not appreciably thicker, stains more sharply and its appearance suggests that during passage of the cuticle it lacked a cell wall. From its subcuticular position immediately beneath the appressorial pore, the fine filament, if it is not directly over the junction of two cells, grows just beneath the cuticle to a position where two epidermal cells meet. In this region it tends to enlarge and from there grows downwards, still in the intercellular region (Fig. 3). Growing in this way, the greatest depth to which hyphae have been traced (in material fixed 143 hours after inoculation) was an angular intercellular space three cells below the cuticle.

The description just given agrees with that of Simmonds in that the infecting hyphae are intercellular but it differs in the extent to which they spread. In the fruits which Simmonds investigated, the banana, mango, and pawpaw, infection appears to have been confined to the development of subcuticular hyphae which, in some cases, were relatively large. The differences observed in the two cases may of course be due simply to the differences in the nature of the hosts studied.

So far as the effect of infection on the host tissue was concerned, there was no evidence of any discolouration of cells or cell walls in unstained preparations. Changes, however, were evident when the thionin-orange G stain was used. In addition to its advantage of staining the hyphae blue in a background of pinkish coloured intercellular material, it also stained blue the granulated and somewhat disorganized cells in the invaded region. On the other hand, healthy uninvaded sections took up the counterstain orange G in such a way as to define the two regions clearly (Plate 1).

IV. DISCUSSION

The objective of a wider investigation, of which this is the initial part, is to elucidate the role which infection by *Colletotrichum* plays in the development of the spotting which occurs in oranges after a period of cool storage. In the first instance, we wanted to ascertain the extent to which different parts of the orange or different groups of oranges might vary in respect to their infection, a fact which is complicated in oranges by the phenomenon of latency.

The results reveal that considerable variations in the degree of infection may occur and that it is possible to give a numerical expression to the prevalence of infection in different parts of the orange, in oranges from different trees and from different districts, and in oranges from the same trees in different seasons.

The significant differences in the infection of oranges picked at the same time from different trees in the same orchard suggest either that inoculum is more abundant on some trees than others or that oranges from some trees are more susceptible than those from others. At this stage it is not possible to present clear-cut evidence in favour of either one of these alternatives but for our purposes the material fact lies in the existence of the differences.

Considerable differences in infection may also occur between oranges taken from the same trees in different seasons, in oranges from different districts, and according to the time when oranges are picked for sampling. These differences, coupled with the absence or low degree of infection occurring in oranges, protected from rain, all point to the importance of weather in determining the degree of infection. However, there is not sufficient data available to indicate the relative importance of the various elements that together constitute the weather. In South Australia it is to be noted that the first significant increase in infection occurs naturally in April or May when the rains of autumn usually commence.

Spray treatments with Bordeaux mixture reduce the degree of infection but the extent of reduction is dependent on times of application. Information was gained from trials where only a limited number of the possible times of application were used. Where the degree of infection was light a single application in April was relatively effective but where conditions for infection are more favourable additional later sprays appear to be necessary for really efficient control.

V. ACKNOWLEDGMENTS

These investigations were part of the programme of a Citrus Preservation Technical Committee set up by the Council for Scientific and Industrial Research with representatives from the Division of Food Preservation and Transport, and the Departments of Agriculture of New South Wales, Victoria, and South Australia. Financial support for the work was afforded by the Council for Scientific and Industrial Research. We are also indebted to the various Departments of Agriculture, especially that of South Australia, for arranging supplies of fruit and for help in other ways.

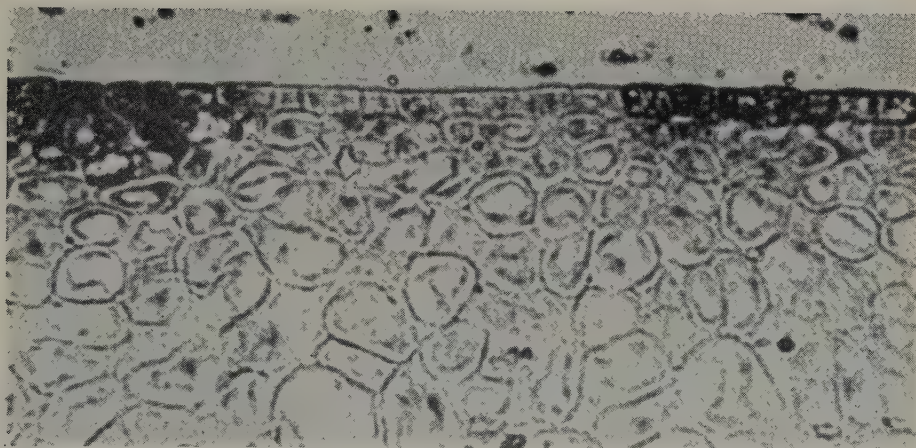
We are also grateful to E. A. Cornish for the considerations referred to earlier and for help generally on statistical problems which arose in the course of the investigation.

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EXPLANATION OF PLATE 1

Staining reaction of tissues invaded by *Colletotrichum*. The darker regions are places at which penetration has occurred. x 450.



ADAM *et al.*— THE ESTIMATION OF LATENT INFECTION IN ORANGES

THE DISTRIBUTION OF ASCORBIC ACID IN THE TISSUES OF INSECTS

By M. F. DAY*

(Plates 1-3)

[Manuscript received November 11, 1945]

Summary

Ascorbic acid has been detected histochemically in many, but not in all tissues of insects. It is particularly abundant in the midgut epithelium and nervous tissue, and is usually confined to the cytoplasm. In two instances nuclei have given a positive reaction for ascorbic acid.

Ascorbic acid fed to the cockroach, *Blattella*, is absorbed by and accumulated in the midgut and caecal epithelium. In *Tenebrio* and *Tribolium* only the mesenteric epithelium absorbs it. Synthetic α -glucosaminic acid which, when fed at the rate of 10 per cent in the diet, produces a scurvy-like condition in rats, produces only minor effects in *Blattella*. This investigation has thrown no light on the functions of ascorbic acid in insects.

I. INTRODUCTION

Although ascorbic acid is not an essential dietary constituent in those insects which have been studied, it has been reported in the tissues of several species, and cockroaches at least can synthesize it. The latter was demonstrated by Willmann, Giroud, and Patummananga (1937) who bred *Blattella* for 15 years on an ascorbic acid-free diet and yet found that these insects contained as much of it (10-15 mg. g.) as newly caught specimens. Joly (1941) found it in the blood of a queen termite and Haydak and Wilson (1941) reported considerable quantities in the honey bee. In the muscles of *Drosophila*, Giroud and Patummananga (1936) reported about three times as much as in the muscles of vertebrates, and Metcalf (1941) found 1.5-10 mg. g. in the malpighian tubules of *Periplaneta*. Contrary to these reports is that of Beppu and Wang (1938) who, using two different calorimetric methods, found no ascorbic acid in *Tenebrio*, *Oryctes*, or *Drosophila*. No explanation has been offered for this, the only negative finding.

As one approach to a study of the function of ascorbic acid in insects, its distribution in the tissues has been examined. The histochemical test employed was that developed by Giroud and Lelander and was used as modified by Toranzo (1938). This technique has only rarely been applied to invertebrates (cf. Smyth, Bingley, and Hill 1945) and not previously, so far as the author is aware, to insects.

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II. MATERIAL AND METHODS

A number of species from various orders have been studied. All were obtained from laboratory colonies or were freshly captured in Canberra. They include adults of the silverfish, *Ctenolepisma longicaudata* Esch., the cockroaches, *Blattella germanica* (L.) and *Periplaneta americana* (L.), and the locust, *Locusta migratoria* (L.), workers of the termite *Nasutitermes exitiosus* (Hill),* larvae and adults of the mealworm, *Tenebrio molitor* L., adults of the flour beetle, *Tribolium confusum* Duval, larvae, pupae, and adults of the blowfly, *Lucilia cuprina* Wied., larvae and adults of the potato moth, *Gnorimoschema operculella* Zeller, larvae of the clothes moth, *Tineola biselliella* Hummel, and workers of the honey bee, *Apis mellifica* L.

Approximately ten individuals of each species were examined except for a queen *N. exitiosus*, of which only one was available. No marked differences between individuals were apparent.

These insects were dissected and the tissues to be studied were immersed for 30 minutes in the dark in 10 per cent. silver nitrate containing two drops of acetic acid per millilitre. The tissues were then washed thoroughly in several changes of distilled water for 30 minutes, placed in 3 per cent. sodium thiosulphate for 30 minutes, washed again in distilled water, and transferred to 70 per cent. alcohol. Dehydration and infiltration were performed in subdued light. Sections of 10 microns were cut, mounted on slides, and lightly stained with eosin or orange G. Some were toned with 1 per cent. gold chloride but generally this did not improve the quality of the preparations.

On the basis of all the available evidence, Barnett and Bourne (1941) have stated that in vertebrates it is "justifiable to assume that the reactions observed are unlikely to be due to reducing substances other than ascorbic acid." This is probably true in insects also, although it is known that melanin granules will reduce silver nitrate in acid solution. However, proper controls readily distinguish these from the ascorbic acid granules. Some evidence for the efficacy of the test can be obtained from the work on ascorbic acid absorption described below. Although Barnett and Fisher's (1943) criticism of the method has been mainly answered by Bourne (1944), the specificity of a histochemical test such as this is extremely difficult to prove. Therefore, although the granules which reduce silver nitrate will generally be referred to as ascorbic acid in this paper, it is recognized that other materials may reduce silver nitrate under the conditions specified.

III. OBSERVATIONS ON THE HISTOLOGICAL DISTRIBUTION OF ASCORBIC ACID IN TISSUES

Ascorbic acid was found to be present in some tissues of almost all insects studied. Its distribution can best be appreciated by reference to the photomicrographs (Plates 1-3).

* This species has been referred to the genus *Eutermes* in previous communications dealing with Australian termites.

(a) *Dermal Tissues*

The cuticle of most insects contains no substance which reduces acid silver nitrate (see Plate 1, Fig. 1). However, the cuticle of the larva of the potato moth, *Gnorimoschema*, contains many very small granules (Plate 1, Fig. 3), and in a few regions of the cuticle of *Tenebrio* larvae a peculiar distribution of larger granules is evident. These are most abundant near the hypodermis and are arranged in rows at right angles to it. One hesitates to suggest that ascorbic acid occurs in the cuticle because of the unusual appearance of the granules (Plate 1, Fig. 2). There is less doubt about its presence in the hypodermal cells of several insects, where it occurs in granules more like those to be described in many other insect tissues. They are most conspicuous in *Locusta* (Plate 1, Fig. 1) in which the cells located at muscle-cuticle attachments contain ascorbic acid in a characteristic pattern. Hypodermal cells which are not attached to muscles contain no such granules. Similar, though less striking deposits of ascorbic acid, are found in certain of the hypodermal cells of the larvae of *Lucilia*, *Gnorimoschema* (Plate 1, Fig. 3), *Tenebrio*, *Nasutitermes*, and *Ctenolepisma*.

(b) *Blood and Circulatory System*

Blood cells of adult *Tenebrio* and of *Periplaneta* were smeared on cover slips and dropped, smear downwards on to the silver nitrate solution. Since poor fixation rendered it impossible to differentiate blood cell types, no more than a general statement of the presence of ascorbic acid in the cells can be given. So far as can be determined from study of the serial sections, positive granules have been seen in the contents of the dorsal vessel of *Lucilia* where haemocytes are absent. This strongly suggests the occurrence of ascorbic acid in the blood plasma of *Lucilia*. No ascorbic acid could be detected in the dorsal vessel except in adult *Lucilia* in which conspicuous granules were present on the surface of the muscle cells of the heart (Plate 3, Fig. 13). Even in this case it appears that the granules are not in the heart muscle, and their presence on the periphery of the cells may, in fact, indicate that they originate from the haemolymph.

(c) *Alimentary Canal*

The gut contains more ascorbic acid than any other tissue, and the greatest part of this is present in the epithelium of the midgut.

The foregut cells usually contain no ascorbic acid. An exception, however, is found in the cells beneath the gizzard teeth of *Locusta*. Here the cytoplasm is packed with granules, while the nuclei contain very few (Plate 3, Fig. 14). In the surrounding muscularis, ascorbic acid is generally confined to the respiratory tissue.

All insects studied have normally a large amount of ascorbic acid in the epithelium of the midgut. Frequently the granules are in greatest concentration at the cell surface so that they outline the cells, as in *Locusta* (Plate 3, Fig. 17); or they may be in greater concentration in the lower part of the cells, as in the queen termite, *Nasutitermes*.

In *Blattella* ascorbic acid is typically located just above the nuclei and occupies the same position as the golgi substance (Plate 1, Fig. 5). In fact it would be difficult to tell whether a preparation demonstrated golgi or ascorbic acid if the technique were not known. After feeding ascorbic acid, both midgut and caecal cells contain a great deal more than usual. It is present in large granules usually on the lumen side of the nucleus; frequently, also, quantities are seen against the muscularis, a condition not usually found in normal insects (Plate 1, Fig. 6).

In the first experiments with *Blattella* the midgut always contained a considerable quantity of ascorbic acid (Plate 1, Fig. 5). Subsequently, however, difficulty was experienced in repeating these early results until it was discovered that, while the colony was first fed largely on cut potato, the food was later changed to a mixture on which the colony thrived even better than on the potato diet. This food was composed of ground whole wheat, dried milk powder, dry yeast, sugar, and fat. Cockroaches fed on this diet reproduced rapidly and appeared healthy in every way. However, the amount of ascorbic acid in the tissues was less than when the insects were fed freshly cut potato, being practically absent from the midgut epithelium, although it was still always present in the fat body. The very low concentration of ascorbic acid in the midgut of these apparently normal insects facilitated the study of the ascorbic acid absorption described below.

The cells of the larval midgut of *Lucilia* contain ascorbic acid scattered through the cytoplasm, but it is absent from the large nuclei (Plate 2, Fig. 10). In *Tribolium* adults the midgut does not normally contain ascorbic acid, but after feeding on ascorbic acid small black granules appear in the midgut epithelium. In the *Tenebrio* adult no ascorbic acid was demonstrated in the tissues. The larva, however, contains some small granules in the midgut epithelial cells, as well as in other tissues. In *Tineola* larvae the ascorbic acid does not appear to be present in the goblet cells and is confined to the perinuclear region of the columnar cells (Plate 3, Fig. 15). Here it may be present in considerable quantity so that the granules coalesce, as apparently also occurs in *Blattella* fed a diet rich in ascorbic acid. It decreases considerably in amount when the larva is starved for 48 hours, although a reduced quantity still remains in the distal part of the cell.

Ascorbic acid is not usually abundant in the cells of the hindgut, but may be present as in the epithelium of *Blattella* (Plate 1, Fig. 4). It is probably absorbed by the hindgut of *Tribolium* since it appears in considerable quantity in the large intestine of these insects when they are fed ascorbic acid.

(d) Excretory System

In *Blattella*, ascorbic acid could not be demonstrated in the malpighian tubules even after feeding on a diet rich in ascorbic acid. The malpighian tubules of most other species contain no ascorbic acid, but *Locusta* provides a striking exception. Here the cytoplasm contains very large granules which are confined

to the centre of the cells, and are bounded by a granule-free layer of cytoplasm. The layer towards the lumen is wider than that towards the haemocoel (Plate 2, Fig. 7). Since the malpighian tubules contain so many granules, including some melanin-like materials, one hesitates to ascribe the positive granules to the presence of ascorbic acid. Nevertheless they are not obvious in control sections.

(e) Storage Tissue

Fat body frequently contains ascorbic acid. Even the histolysing pupal fat body present during the first few days of the life of the adult *Lucilia* may show granules scattered among, and frequently adherent to, the large fat body granules.

In *Blattella*, ascorbic acid is never found in the cells containing the bacteroids, but is present in large finger-like masses in some of the other fat body cells (Plate 2, Fig. 8). This shape of the granules is unusual. In starved *Blattella* the ascorbic acid remains in the fat body after it has entirely disappeared from the midgut.

(f) Muscle Tissue

The muscles of insects are more variable than other tissues with regard to their ascorbic acid content. Muscles which show conspicuous granules may be next to those which have none. In the gizzard of *Blattella* the muscles contain some granules and, in addition, the tracheoles which ramify through the tissue are deeply impregnated.

The flight muscles of *Lucilia* contain more ascorbic acid than other muscles studied. When viewed in either transverse or longitudinal section the granules seem to be uniformly scattered through the sarcomeres (Plate 2, Fig. 9).

(g) Respiratory Tissue

Fine tracheoles frequently give a positive reaction for ascorbic acid. They are conspicuous in the nerve ganglia of *Locusta* and the crop muscles of *Blattella*. The epithelial cells of larger tracheal trunks entering the brain of *Lucilia* larvae sometimes contain a few scattered granules.

(h) Nervous Tissue

The nerve ganglia of all insects studied contain ascorbic acid. It is present both in the cytoplasm of the nerve cell bodies and in the central fibrous mass. In *Locusta* it is particularly abundant in the neurilemma and it may extend out into the nerve axons as in *Lucilia*. Nuclei are invariably without ascorbic acid in nervous tissue, but tracheae in nervous tissue may be particularly well impregnated. No illustration of ascorbic acid in nervous tissue is included since resolution of the cell boundaries in photomicrographs is difficult.

(i) Glandular Tissue

The salivary glands are completely negative for ascorbic acid although the tracheae supplying them may sometimes give a weak, positive reaction. The corpus allatum of the honey bee is completely negative but the corpus cardiacum of *Locusta* contains conspicuous granules especially on the periphery of the cells (Plate 3, Fig. 16).

(j) Organs of Intermediary Metabolism

In the majority of species examined the pericardial nephrocytes show no accumulations of ascorbic acid, although a little is present in those of *Lucilia* (Plate 3, Fig. 13). The oenocytes of *Lucilia* have been found to be negative; but in *Locusta* the oenocytes are markedly positive and their nuclei also show a strong reaction (Plate 2, Fig. 12).

(k) Reproductive System

Both male and female reproductive organs may contain relatively large amounts of ascorbic acid. The developing ova of *Periplaneta* contain it at certain stages. The central ovum in Plate 2, Figure 11, contains considerable quantities while the cytoplasm of the larger ovum at the upper right hand corner of the photograph contains none. The nucleolus of the smaller ovum gives a positive reaction. The nucleoli in the ova of a queen termite, *N. exitiosus*, are negative, although the cytoplasm contains considerable quantities (Plate 3, Fig. 18). It will be noted that the granules are concentrated at one side of each egg. The spermathecal duct of a female *Tenebrio* is found to contain ascorbic acid but, in general, the accessory glands contain smaller quantities than the gonads.

IV. THE EFFECT OF ASCORBIC ACID DEFICIENCY IN BLATTELLA

Since *Blattella* in the stock cultures appeared normal when their tissues contained markedly less ascorbic acid than when they were fed on potato (see above), it seemed desirable to determine whether scorbutic effects could be produced in this insect. Since *Blattella* synthesizes its vitamin C requirements, the only available method of investigation was to feed a specific antagonistic substance. It has been suggested that *d*-glucoascorbic acid (2,3-enediol-*d*-glucoheptono-1,4-lactone)* is such a material. This was fed to mice and cotton rats by Woolley and Krampitz (1943), and was found to produce in them within seven days a clinically striking, scurvy-like condition. A histological examination of insects which had ingested comparatively large quantities of pure *d*-glucoascorbic acid, revealed few striking changes from the normal histology. Conspicuous basophilic granules were found in the distal regions of the epithelial cells of the large intestine. These may be found in normal insects but are generally less distinct. There was a marked enlargement of the pericardial nephrocytes, some of which contained basophilic granules not found in normal *Blattella*. It is not possible to compare this result with previous data, the only report of pathological changes in insect tissues due to avitaminosis being that of Swamy and Sreenivasaya (1942), who dealt with the effects of vitamin B complex deficiency on *Corcyra*.

In chronic feeding experiments adults were raised from the egg on ground-up food containing 10 per cent. *d*-glucoascorbic acid. The first adult was produced in 47 days at 30°C., only three days after the first was produced in the controls

* One gram of this material was made available through the kind cooperation of Dr. Philip P. Gray of the Wallerstein Laboratories, New York.

fed a normal diet. Histological examination revealed that the pericardial nephrocytes were enlarged as in the acute feeding experiments. Chemically, *d*-glucoascorbic acid would be expected to reduce acidified silver nitrate solutions, and since in the tissues it apparently gives the same reaction in the acid silver nitrate method as ascorbic acid, it was difficult to determine whether the distribution or amount of the latter was altered in the experimental group. But certainly there were no marked changes.

The recent work of Gould (1948), received after these results had been obtained, suggests that the condition produced in rats by feeding *d*-glucoascorbic acid is not scurvy, and that the effects observed may not be an example of ascorbic acid antagonism.

V. THE ABSORPTION OF ASCORBIC ACID FROM FOOD

The observation that ascorbic acid could be made visible in the midgut epithelium when insects were fed a diet rich in it, has made possible the study of the absorption of this substance (Plate 1, Figs. 5 and 6). After the whole midgut had been fixed in the silver nitrate solution and subsequently treated according to the method given above, it was cut longitudinally and mounted whole. It was then possible to study the absorption of ascorbic acid over the entire length of the midgut and it could be seen that, although the cells of the midgut epithelium of *Blattella* appear homomorphous following the usual histological techniques, they do not all absorb ascorbic acid in the same way. In the anterior part of the midgut the amount is small, and the granules, though minute, are evenly dispersed in the distal cytoplasm. Then follows a region, about three-quarters of the length of the gut, in which both the number and the size of the granules are larger. In this region some of the granules outline the cell walls. Finally, there is a short region anterior to the entrance of the malpighian tubules where the number of granules is less and their size smaller. This region can readily be distinguished from the anterior region since the granules are confined to the periphery of the cells, a condition observed in a variety of tissues in different insects. In *Periplaneta* differences in absorption of ascorbic acid in different regions are less marked, but are discernible.

The histological observations on the absorption of ascorbic acid in the midgut and caeca of *Blattella* were confirmed by a quantitative chemical estimation. Four groups, each of five adult *Blattella*, previously fed on the artificial diet, were fed for one week, respectively on (i) the artificial diet, (ii) cut potato, (iii) ground ascorbic acid tablets (Mead's 25 mg. tablets), (iv) the artificial diet + 10 per cent. *d*-glucoascorbic acid. The midguts and caeca of each group were ground in centrifuge tubes with 1 ml. of metaphosphoric acid and a little sand. The brei was then centrifuged and the ascorbic acid in 0.4 ml. of the supernatant was estimated by the method of Pecover (1947). The results (Table 1) indicate that, although the amount was fairly low in all groups, the ascorbic acid fed group contained a significantly greater amount of ascorbic acid than either the potato fed group, or that fed the artificial diet. The high

value for the *d*-glucoascorbic acid fed group confirms the conclusion that the substance has in the tissues the strong reducing properties of ascorbic acid. Since it was fed in admixture with the artificial diet, more of it was presumably ingested than of the ascorbic acid itself.

TABLE 1
ASCORBIC ACID (MG./G.) IN *BLATTELLA* MIDGUT

Artificial Diet	Potato	Ground Ascorbic Acid Tablet	Artificial Diet + 10% <i>d</i> -Glucoascorbic Acid
0.1	0.2	0.7	6.0

No ascorbic acid could be detected in the midgut of the normal flour beetle *Tribolium*, or the adult mealworm *Tenebrio*. When large amounts were fed in the diet, it was demonstrated only in the intercryptal cells. Apparently the cells of the crypts are not absorptive. Duspiva's (1939) study of the distribution of enzyme production in the epithelium of *Dytiscus* has shown that the crypts produce the protein digesting enzymes, dipeptidase and aminopolypeptidase, but most of the tryptic proteinase is produced by the intercryptal epithelium. It would appear, therefore, that the cells of the latter zone may be both secretive and absorptive.

TABLE 2
NUMBER OF MITOSES IN MIDGUT CRYPTS OF *TENEBRIO MOLITOR*. EACH FIGURE IS THE AVERAGE COUNT OF 30 CRYPTS

	Controls. Injection with Insect Ringer			Injection with 10 ⁻³ M Ascorbic Acid			Injection with 10 ⁻³ M <i>d</i> -Glucoascorbic Acid			
½ hr. after injection	2.9	3.6	2.6	3.0	3.8	2.7	4.4	3.3	2.8	3.1
Average		3.0			3.2			3.4		
1 hr. after injection	2.3	3.6	3.7	3.3	3.6	2.6	2.4	2.7	3.1	2.9
Average		3.0			3.0			2.9		

VI. THE SIGNIFICANCE OF ASCORBIC ACID IN INSECT TISSUES

It is a remarkable fact that, in spite of the great amount of work done on ascorbic acid in vertebrates, there is still no indication of its biochemical role. In discussing this subject Shapiro (1948) has recently demonstrated that ascorbic acid has, in physiological concentrations, an effect on the rate of cleavage in *Arbacia* eggs. The possibility of its affecting mitosis in insects has been tested by injecting it into the haemocoel of *Tenebrio molitor* and counting the numbers of dividing cells, according to a method soon to be described. The effect of *d*-glucoascorbic acid injections was also studied. Approximately 50 µg. of 10⁻³M solution in insect Ringer solution was injected into each insect. The results are given in Table 2.

None of these differences is significant, and it is concluded that, in the concentration employed, no effect on the mitosis of the midgut regenerative cells of *Tenebrio* is exerted by either ascorbic acid or *d*-glucoascorbic acid.

VII. DISCUSSION

Considerable evidence that the histological method employed does demonstrate ascorbic acid in the tissues, has been obtained from the experiments in which normal and ascorbic acid-fed insects were fixed simultaneously. There is some question, however, of the efficiency of the method in localizing the substance within the cells. The accumulation observed at cell surfaces, as in midgut epithelia, for example (Plate 3, Fig. 17), may be real, but may represent only the lines of diffusion of the fixing fluid into the cells. This is, indeed, suggested in an example of the termite ovary (Plate 3, Fig. 18) where the substance is demonstrated towards the same side in each cell. The accumulation of ascorbic acid in the golgi zone, as pointed out by Bourne in vertebrate tissues, is only occasionally found in insects (as in Plate 1, Fig. 5, and Plate 2, Fig. 10). However, the comparative rarity of its occurrence within the nucleus, with the striking exceptions of its presence in the nucleolus of the *Blattella* ovum, and in the nuclei of the *Locusta* oenocytes, undoubtedly represents real differences in the distribution of ascorbic acid.

The results of the histological localization of ascorbic acid agree with the scanty chemical data available. Thus its presence in *Lucilia* blood plasma may be compared with Joly's detection of it in the blood of the queen termite, but Metcalf's estimation of ascorbic acid in the malpighian tubules of *Periplaneta* has not been confirmed by the histological method. Whether this is due to inhibitory or masking substances, such as are reported in the vertebrate liver, has not been determined. The results of the chemical determinations described above confirm the histological findings.

Neither the distribution of ascorbic acid in the tissues nor the study of its effect on the mitosis of regenerative cells in the midgut of *Tenebrio* offers any hint of its possible functions in insects. The experiments with *d*-glucoascorbic acid lead to one of two conclusions; either this substance at the dosage employed does not completely antagonize ascorbic acid in the tissues (compare the spontaneous cures recorded by Woolley and Krampitz (1943)), or ascorbic acid does not play the significant role in insects that it does in vertebrates. In view of the recent work of Gould (1948) the former alternative appears likely. It may even be possible that ascorbic acid is an unavoidable metabolic by-product in insects, although further work is necessary to prove this hypothesis.* Nevertheless, the known locales of action of vitamin C in vertebrates (see Höjer 1924), such as connective tissue and sites of calcification, either do not exist in insects, or occur in forms greatly different from those of vertebrates.

* It is interesting that Fox (1948) has made a similar suggestion with regard to haemoglobin in *Daphnia*.

One further observation should be made. The digestion of keratin in the midgut of the larva of the clothes moth, *Tineola biselliella* Hum., has been shown by Linderström-Lang and Duspiva (1936) to be dependent on an unusually low oxidation-reduction potential in this region of the gut, but they have not suggested what systems might be responsible for this condition. It seemed possible at the outset of the study that the ascorbic acid redox system could play a contributory role. In view of the absence of any outstanding difference between the amount or the distribution of ascorbic acid in *Tineola* tissues, especially in the midgut epithelium (Plate 3, Fig. 15), it is not likely that the ascorbic acid system plays any different part in the physiology of *Tineola* from that which it plays in other insects.

VIII. ACKNOWLEDGMENTS

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EXPLANATION OF PLATES 1-3

PLATE 1

- All photomicrographs taken with Leica IBSO attachment x 96 objective, x 8 ocular. Sections 10 microns, Leblond AgNO₃ method to demonstrate ascorbic acid. Magnification x 620.
- Fig. 1.—*Locusta migratoria*, section of abdominal cuticle, hypodermis containing ascorbic acid and muscle, the latter barely differentiated. The clear areas in the hypodermis indicate the presence of nuclei. The dark "epicuticle" is due to pigment.
- Fig. 2.—*Tenebrio molitor* larva, section of abdominal cuticle and hypodermis. The positive granules are arranged perpendicular to the hypodermis.
- Fig. 3.—*Gnorimoschema operculella* larva, section of cuticle, darkly staining hypodermis and underlying fat body. Note small darkly staining inclusions in the cuticle, and the large positive granules scattered through the fat body.
- Fig. 4.—*Blattella germanica*, T.S. large intestine, chitinous lining separated from epithelial cells which contain ascorbic acid. A few granules also present in muscularis.
- Fig. 5.—*B. germanica*, T.S. midgut normal adult fed a diet including potato. Almost all ascorbic acid in the position of the golgi substance, that is, distal to the nucleus.
- Fig. 6.—*B. germanica*, T.S. midgut of adult fed ascorbic acid. Compare with Figure 5 and note increased positive reaction distal to the nuclei, and presence of some ascorbic acid at the proximal pole of the cells.

PLATE 2

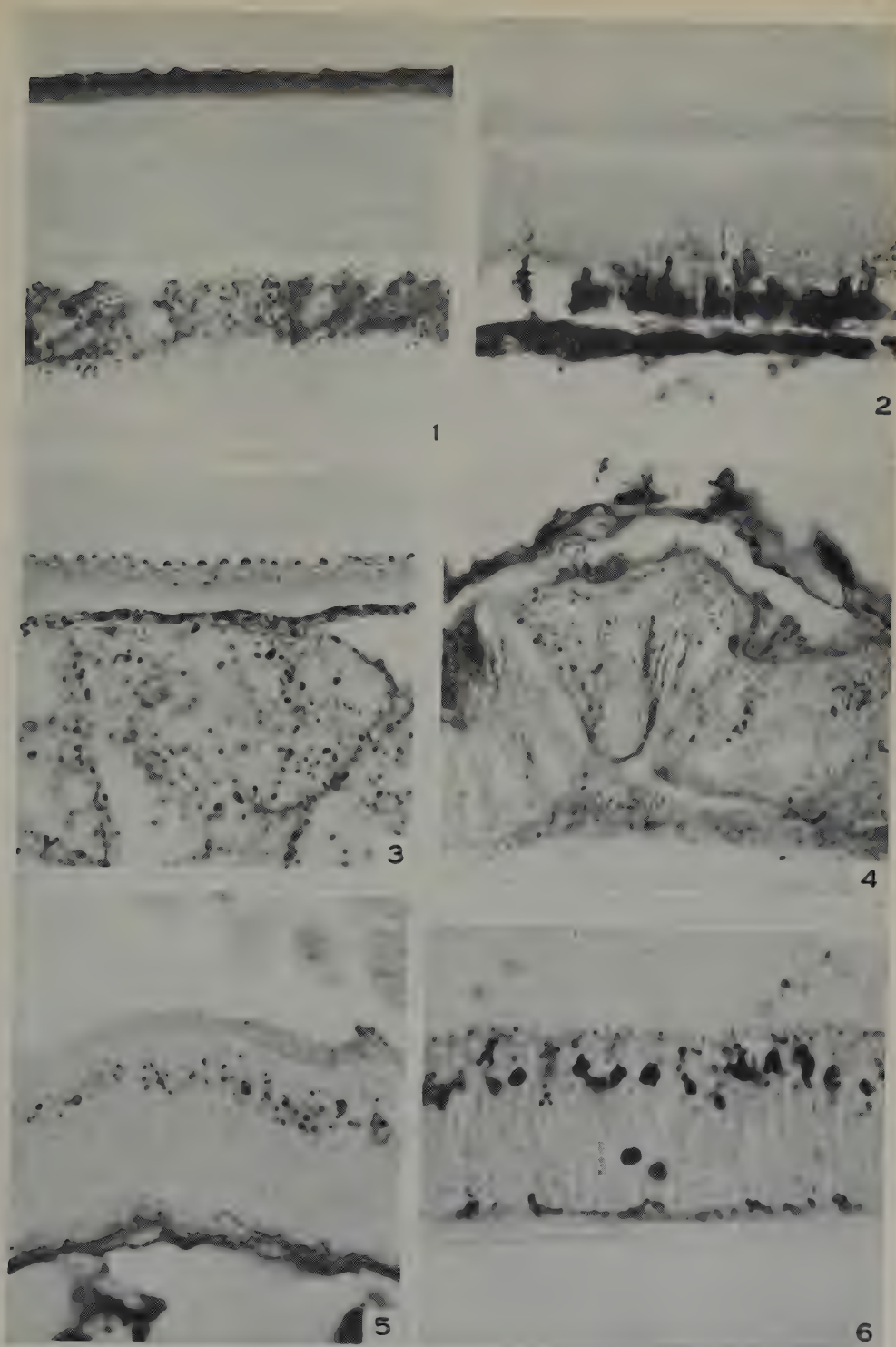
All photographs as in Plate 1.

- Fig. 7.—*Locusta migratoria*, T.S. malpighian tubules. Note large granules probably ascorbic acid in central cytoplasm, but not in the nuclei.
- Fig. 8.—*B. germanica*, fat body showing ascorbic acid in masses in peripheral fat cells, never in central cells containing bacteroids.
- Fig. 9.—*Lucilia cuprina*, T.S. muscle. Note irregular distribution of ascorbic acid among sarcomeres.
- Fig. 10.—*Lucilia cuprina*, T.S. midgut. Ascorbic acid fairly uniformly dispersed through the cytoplasm, but absent from the nucleus.
- Fig. 11.—*Blattella germanica*, section of young ovum. Ascorbic acid granules abundant in the cytoplasm, absent from the nucleus except the centrally located nucleolus.
- Fig. 12.—*Locusta migratoria*, section of fat body and oenocyte. The cytoplasm and the nucleus of the oenocyte contain much ascorbic acid.

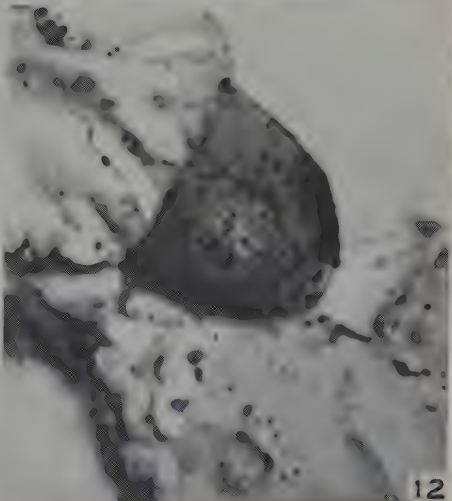
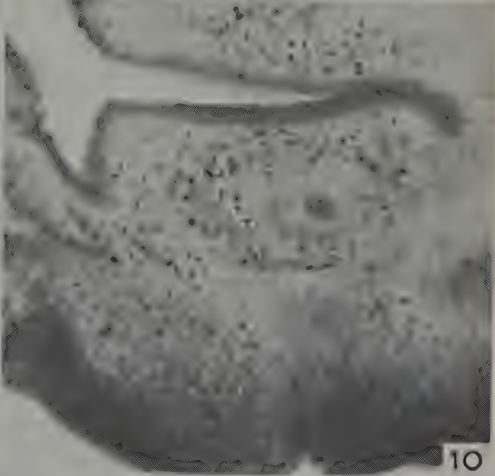
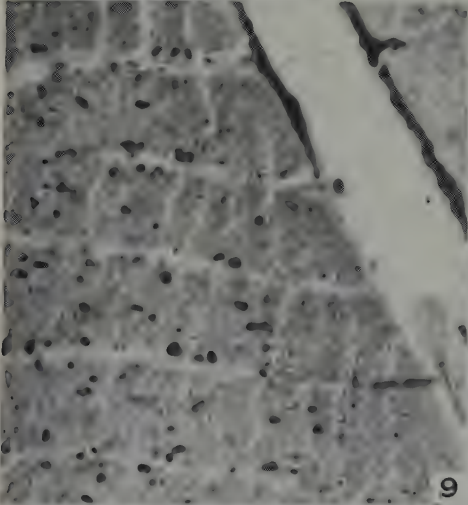
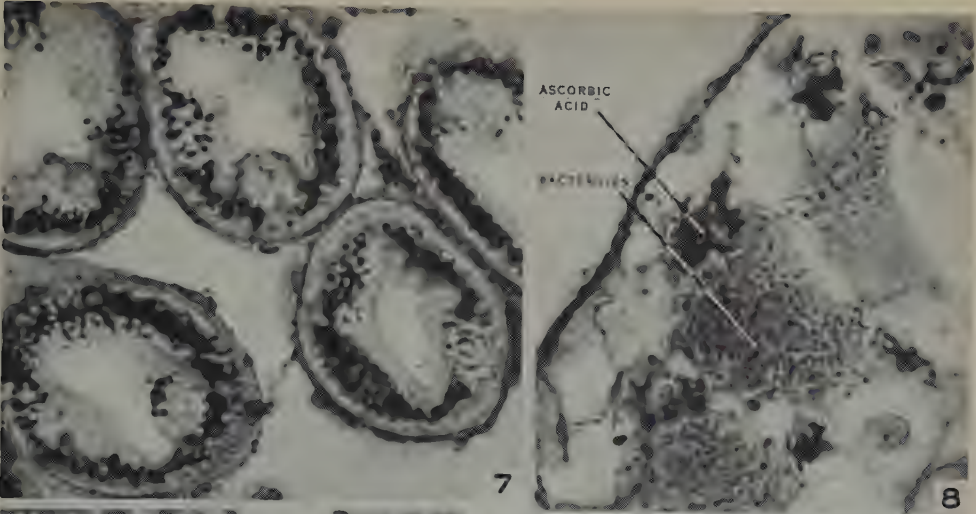
PLATE 3

All photographs as in Plate 1, except x 96 objective, x 5 ocular. Magnification x 380.

- Fig. 13.—*Lucilia cuprina*, T.S. abdomen. Cuticle contains pigment, no ascorbic acid. The hypodermis contains a little. A part of a section of the heart is on the left. Its muscular walls contain ascorbic acid. Pericardial nephrocytes contain some positive granules.
- Fig. 14.—*Locusta migratoria*, L.S. gizzard. To the left is the muscularis, with positive ramifying tracheae. Large epithelial cells are very rich in ascorbic acid but the nuclei contain none.
- Fig. 15.—*Tineola biselliella*, L.S. midgut. Ascorbic acid in the columnar cells, but not in the goblet cells of the epithelium.
- Fig. 16.—*Locusta migratoria*, T.S. corpus cardiacum. Granules of ascorbic acid delineate cell borders of glandular part of the organ. The nervous part, not photographed, also contains some positive granules.
- Fig. 17.—*Periplaneta americana*, T.S. midgut, showing positive granules outlining epithelial cells. The regenerative cells contain very little.
- Fig. 18.—*Nasutitermes exitiosus* queen, L.S. ovariole. Cytoplasm of each ovum contains conspicuous granules mainly confined to one side of the cell.



DAY,—THE DISTRIBUTION OF ASCORBIC ACID IN THE TISSUES OF INSECTS





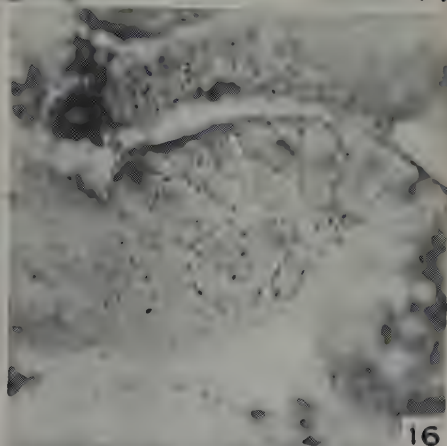
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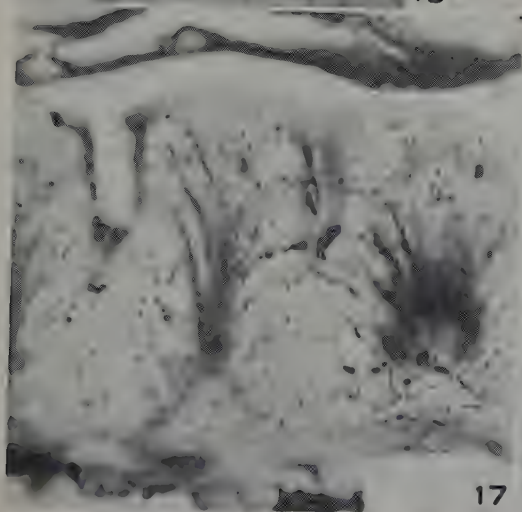
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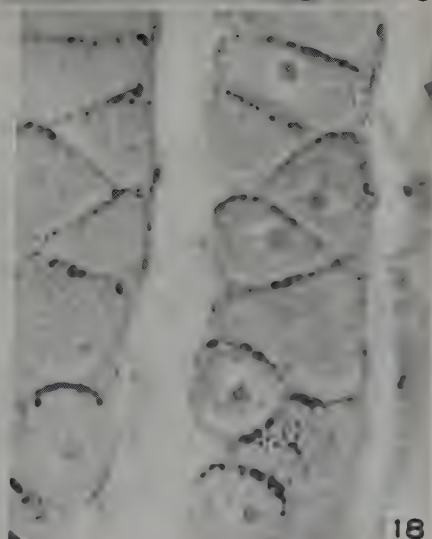
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THE DISTRIBUTION OF ALKALINE PHOSPHATASE IN INSECTS

By M. F. DAY*

(Plates 1-3)

[Manuscript received November 11, 1948]

Summary

The distribution of alkaline phosphatase in insect tissues has been studied by a histochemical technique. The enzyme is widely distributed in the alimentary tract, storage tissue, nervous tissue, in parts of the reproductive system, in certain muscles, and some glands. It thus appears to be involved in a variety of functions. Many examples of both the histological and cytological distribution are explained by the relation of the enzyme to mechanisms of transport across cell boundaries. But a function of this kind is not evident in such sites as muscles and nerves. The presence of deposits of inorganic phosphate in the alimentary tract and malpighian tubules of some species is recorded.

I. INTRODUCTION

The tremendous current interest in the biochemistry of phosphates and phosphatases is largely due to their unique role in energy transfer and in enzymatic syntheses (Kalckar 1947). A special impetus to the study of phosphatases was given by the discovery of comparatively simple methods for their histological detection. Yet there is only one brief report (Bradfield 1946) on the localization of alkaline phosphatase in insects, which concluded that the enzyme is located in cells most active in the synthesis of fibrous proteins. In vertebrates the phosphatases are widely distributed (see for example, Gomori 1941; Bourne 1943a) and this and other evidence (Moog 1946) suggests that they are concerned with other functions as well. Since a wider distribution of phosphatases in insect tissues than that reported by Bradfield has been indicated by several microchemical studies (Drilhon 1943; Drilhon and Busnel 1945; Nakamura 1940), it seemed desirable to investigate in greater detail the localization of the enzymes in a number of insect species.

II. MATERIAL AND METHODS

Tissues of a number of insects were examined by the well-known Gomori-Takamatsu technique, and the schedule recommended by Gomori (1941) was followed in detail. Sodium glycerophosphate (B.D.H.) was used as the substrate. The species examined were: *Ctenolepisma longicaudata* Esch., *Blattella germanica*

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(L.), *Periplaneta americana* (L.), *Locusta migratoria* (L.), *Tenebrio molitor* (L.), *Tineola biselliella* Hum., *Pieris rapae* (L.), and *Lucilia cuprina* Wied.

No differences were observed in the distribution of alkaline phosphatase between any of the several specimens (at least three) of each species examined. A large number of *B. germanica* were studied and no example of variation in the distribution of the enzyme was found.

The specificity of the technique has been thoroughly discussed by Danielli (1946). He showed that, provided controls omitting the substrate are stained simultaneously to distinguish preformed phosphate, there can be no doubt of the specificity of the reaction.

III. OBSERVATIONS

(i) *General*.—The majority of insect tissues have, in one species or another, been found to be positive for alkaline phosphatase. Exceptions are the dermal tissues, the heart, and the pericardial nephrocytes (Table 1). Usually the nuclei gave a stronger reaction than the cytoplasm. Nucleoli frequently were positive also in the controls indicating the presence of inorganic phosphate. Usually the enzyme was confined to one region of the cytoplasm, especially the periphery or free border of the cell. The reaction was generally diffuse and probably never confined to cytoplasmic granules. Where granules occurred they were always positive in the controls also, indicating the presence of inorganic phosphate. It was, of course, possible that some enzyme was also located in the granules, but its presence was obscured.

A comparison between the distribution of alkaline phosphatase and of ascorbic acid in insects (Day 1949) reveals that these two substances were rarely present together in cells. When they did occur together in the same cells, e.g. in fat body of *Blattella*, they generally occupied different positions in the cells. Exceptions to this generalization were found in the nervous tissue.

(ii) *Dermal Tissue*.—Hypodermal tissue, except for sense cells (see below), was negative in all species studied. Occasional nuclei of the hypodermis of *Ctenolepisma* and of *Locusta* were positive but the majority contained no demonstrable alkaline phosphatase.

(iii) *Alimentary Canal*.—Foregut. The epithelial cells of the pharynx of several insects gave a strongly positive reaction. In *Ctenolepisma* (Plate 1, Fig. 1) the pharyngeal epithelium in the head region stained uniformly, the reaction being especially marked in a sense organ just below the supraoesophageal ganglion. In *Locusta* (Plate 1, Fig. 2) the epithelium was uniformly stained, while in *Tenebrio* only the lumen border of the epithelium was positive.

Midgut. In many species there was no obvious reaction in the midgut epithelium, but in *Ctenolepisma* the cytoplasm was faintly positive, the nuclei strongly so, and in *Blattella* a few small positive areas were seen between the nucleus and the striated border in both the epithelium of the midgut and its caeca.

TABLE 1
HISTOLOGICAL DISTRIBUTION OF ALKALINE PHOSPHATASES IN INSECT TISSUES

Species	Dermal Tissues Blood Heart	Alimentary Canal			Malpighian Tubules	Tissue				Glands (Salivary and Silk)	Oenocytes	Reproductive Systems
		Foregut	Midgut	Hindgut		Storage	Respiratory	Muscle	Nervous			
<i>Ctenolepisma</i>	N	+	+	+	-	N	-	+	+			
	Dermal tissues only	(Plate 1, Fig. 1)										
<i>Blattella</i>	-	+	Especially muscularis	(Plate 3, Fig. 13) Rectum (Plate 2, Fig. 7)	+	(Plate 2, Fig. 8)	-	+	+	+	-	+
					Muscles rarely (Plate 3, Fig. 15)					(Plate 2, Fig. 11)		(Plate 2, Fig. 10)
<i>Periplaneta</i> embryo	-	-	-	-	-	+	-	-	+			+
<i>Periplaneta</i> adult	-	+	(Plate 1, Fig. 3)		-	-	-	-	+			+
<i>Locusta</i>	N	+	-	+	+	(Plate 2, Fig. 9)	In rectum only	+	+			+
	Dermal tissues only	(Plate 1, Fig. 2)		Rectum (Plate 3, Fig. 14)	-				(Plate 2, Fig. 12)			
<i>Tenebrio</i> larva	-	-	-	+	-	-	-	+	-			
<i>Tenebrio</i> adult	-	-	Faintly positive reaction	+	+	-	-	-	-		-	
<i>Pieris</i> larva	-	N	N			N	N	+	+	+	+	
										(Plate 3, Fig. 16)		
<i>Timeola</i> larva	-	-	Columnar cells only		+	-	-	-	-	+		
<i>Lucilia</i> larva	-	+	(Plate 1, Fig. 4)	+	-	-	-	+	+			
<i>Lucilia</i> pupa	-	+	(Plate 1, Fig. 5)		-	-	-	-	+			
<i>Lucilia</i> adult	-	+		+	+	-	-	-	+	+	-	+
				Especially rectal glands (Plate 1, Fig. 6)								

- indicates alkaline phosphatase absent. + indicates alkaline phosphatase present. No mark indicates the tissue was not studied. N indicates the positive reaction is confined to the nucleus.

The most unexpected location for alkaline phosphatase found in this investigation was the circular muscularis of the anterior third of the midgut of *Blattella* and of *Periplaneta* (Plate 1, Fig 3). The region was distinctly limited and the reaction was much stronger than elsewhere in the gut. Posteriorly the positive reaction ceased without any change in the histological appearance of the muscles following normal staining procedures. Only the circular muscles, not the longitudinal muscles in contact with them, gave the reaction. It is interesting to note the positive muscles occurred in the same region as the nerves from the stomodeal nervous system, which innervate only the anterior end of the midgut. In the *Tenebrio* adult the intercryptal epithelium and the cells of the crypts themselves gave a faintly positive reaction. In *Tineola* larvae some columnar cells of the epithelium were faintly positive, while in the *Pieris* larva the nuclei but not the cytoplasm of the same cells also gave a positive reaction. In *Lucilia* larvae the midgut (Plate 1, Fig. 4) cells gave a strong positive reaction on their lumen border and the chromatin of the polytene nuclei were also intensely positive (cf. Krugelis 1945). In the pupa (Plate 1, Fig. 5) the reaction at first was confined to the periphery of the cell but appeared to spread through the foamy cytoplasm as the cells prepared to undergo the changes of metamorphosis. The midgut of the adult *Lucilia* was negative.

Hindgut. The epithelium of the hindgut may either be positive or negative for alkaline phosphatase, depending on the species. A negative reaction was observed in the adult *Tenebrio*, and the larvae of *Tineola* and *Lucilia*, while a positive reaction was found in *Ctenolepisma*, *Blattella*, *Locusta*, and the larva of *Tenebrio*. In *Blattella* and *Tenebrio* some cells of both the large and small intestines gave a strong reaction on the inner cell border, with a less marked reaction in the nuclei (Plate 3, Fig. 13). In the *Locusta* hindgut, which has a thick chitinous intima, the entire cytoplasm of the epithelial cells was positive, but the muscularis contained no alkaline phosphatase.

In the rectum of *Blattella* (Plate 2, Fig. 7) the "rectal glands" were negative, but the thin epithelium connecting the "glands" was strongly positive. This positive region includes the epithelium up to the chitinous ring which surrounds each rectal gland. The concentration of the enzyme in the cells referred to by Wigglesworth (1933) as "vestigial" suggests that these cells do, in fact, serve a function other than the mere joining of the "rectal glands." It is possible that it is these, rather than the "gland" cells which absorb water from the faecal pellet, as it is formed.

The alkaline phosphatase in the rectal glands of *Locusta* presented a very unusual appearance since the enzyme was confined to the region of the intracellular tracheoles characteristic of some Locustidae. These tracheoles arise from tracheal end cells, the presence of which was observed by Tietz (1923) in *Dissosteira* and by Chauvin (1941) in *Schistocerca*, but which have never been adequately described. The rectum of *Locusta*, like that of most insects, is well tracheated. Large trunks pass through the muscularis and penetrate between

the cells of the epidermal layer of the rectal glands. At this point there is a nucleated tracheal end cell and the trachea gives off a number of tracheoles which become intracellular and run back towards the muscularis. The tracheoles are arranged like the ribs of an umbrella and these and the tracheal end cell both gave a positive reaction for alkaline phosphatase (Plate 3, Fig. 14). This was the only case observed in which the enzyme was associated with the respiratory tissue.

The rectum in the larva of *Tenebrio*, the larva of *Anthrenus*, and the adult honey bee gave no positive reaction.

The rectal papillae of *Lucilia* contained a high concentration of alkaline phosphatase mostly confined to the periphery of the large papillar cells (Plate 1, Fig. 6). The intracellular tracheae (Graham-Smith 1934), unlike those of the *Locusta* rectal gland, gave no positive reaction for alkaline phosphatase.

(iv) *Malpighian Tubules*.—Intracellular granules of phosphate occurred in the malpighian tubules of a number of insects including *Blattella* (Plate 3, Fig. 15), *Periplaneta* (Plate 2, Fig. 8), *Locusta*, adult *Tenebrio*, *Apis* workers, and *Lucilia* larvae, pupae, and adults. In the larvae and pupae of *Lucilia* there were granules in the tubule lumen as well as in the cytoplasm. The cytoplasm may also give a positive reaction for alkaline phosphatase, as in some regions of the tubules of *Blattella* (Plate 3, Fig. 15), and as reported by Bradfield (1946) in *Cossus*. More frequently only the striated border of the tubules was positive. In *Tineola* the striated border was positive in those regions of the tubules which envelop the rectum, but was less so in other regions, while in the worker honey bee the tubules were positive in some regions and negative in others. In *Lucilia* adults the striated border was positive over the greater part of the tubule (Plate 1, Fig. 6).

(v) *Fat Body*.—In most species studied the fat body contained no alkaline phosphatase. In some lobes of this tissue in *Blattella* and *Periplaneta* (Plate 2, Fig. 8) there were definite positive areas, usually on the periphery in the former species, but mostly contiguous with the bacteroid cells in *Periplaneta*. In *Locusta* the cytoplasm of the fat body was positive and the nuclei strongly positive (Plate 2, Fig. 9). In no other species examined did the fat body contain large amounts of alkaline phosphatase. Only the nuclei stain in *Pieris* larvae and in the larvae and pupae of *Lucilia*. In pupae of the latter species some of the globules of the fat body cytoplasm gave a faint reaction.

(vi) *Respiratory Tissue*.—The trachea or tracheal epithelium was negative in all insects studied. Some nuclei may give a positive reaction as in *Pieris*. The positive intracellular tracheolar system in *Locusta* rectal glands has already been described.

(vii) *Muscle Tissue*.—Muscles in almost all species gave no reaction for alkaline phosphatase. A few, as in the thorax of *Ctenolepisma* and *Lucilia* and some circular muscles of the *Pieris* hindgut, may be faintly positive, but are weakly so in comparison with other tissues of these species.

This makes all the more interesting those cases in which alkaline phosphatase is abundant in muscles, namely, the circular muscles of the anterior end of the midgut of *Blattella* and *Periplaneta* (Plate 1, Fig. 3), some spiral muscles of *Blattella* malpighian tubules (Plate 3, Fig. 15), and a small group of muscles of the female genitalia of *Blattella*.

(viii) *Nervous Tissue*.—Generally the nervous tissue is strongly positive for alkaline phosphatase. For example, sense organs of the leg and pharynx (Plate 1, Fig. 1) of *Ctenolepisma* were positive, as was the cytoplasm of the cuticular sense organs of *Locusta*. The brain was positive in all insects studied and in most species the ganglia and ventral nerve cord were rich in the enzyme. Plate 2, Figure 12, shows a region of the brain of *Locusta*. The nuclei and optic fibre tracts are positive. The ventral nerve cord of *Locusta* was an exception and gave no reaction. The nerve cell bodies of the central nervous system and fibre tracts were the only positive regions in the embryo of *Periplaneta*. The neurilemma was negative.

(ix) *Glands (except Accessory Sex Glands)*.—Bradfield (1946) reports a positive reaction in the spinning glands of the moth, *Cossus cossus*. This has been confirmed in the silk glands of *Pieris* and *Tineola*. The salivary glands in *Ctenolepisma* and *Blattella* (Plate 2, Fig. 11) contained some positive regions as did the pygidial glands of the male *Blattella*. The enzyme in *Blattella* salivary glands was present in both the peripheral cells and the granular cells, but was especially strong in all nuclei and in the intralobular ducts.

The corpora allata and cardiaca were negative in all species studied. However, Arvy and Gabe (1947) record a faint positive reaction in the corpus allatum of *Chironomus*. The properties of the phosphatases in *Drosophila* salivary glands have been investigated by Doyle (1947).

(x) *Oenocytes and Pericardial Nephrocytes*.—The oenocytes in most species are free from alkaline phosphatase, except for the nuclei which may show slight positive reactions (*Locusta*, *Tenebrio*, *Lucilia*). In the larva of *Pieris* the reaction was strongly positive (Plate 3, Fig. 16). A rather unusual cytological distribution was evident in this case. The peripheral cytoplasm and the irregularly-shaped nuclei had a high concentration of enzyme but surrounding each nucleus there was a clear area frequently almost devoid of enzyme. Pericardial nephrocytes were negative in all species studied.

(xi) *Sex Organs*.—The spermatocytes may be faintly positive in *Periplaneta*. Other gonadal tissue of this species and male gonadal tissue of all other species were negative. Male accessory glands gave a positive reaction in *Blattella* (Plate 2, Fig. 10), *Locusta*, and *Lucilia*. Female accessory glands were positive in *Blattella* but have not been studied in other species.

IV. EFFECT OF ASCORBIC ACID ON ALKALINE PHOSPHATASE

There are many reports of the effect of scurvy on alkaline phosphatase in vertebrates. Thus, for example, in a survey of 241 cases of scurvy in man, Dogramaci (1946) found serum alkaline phosphatase to be abnormally low, and

Bourne (1943*b*) concluded from his study of calcification in guinea pigs that ascorbic acid may act in the formation or stabilization of alkaline phosphatase. But he found (1943*a*), as did Harrer and King (1941), that kidney alkaline phosphatase was only slightly changed in the scorbutic guinea pig. Since the effects of feeding the ascorbic acid antagonist, *d*-glucoascorbic acid, to *Blattella* have recently been investigated (Day 1949), it seemed worthwhile to study these insects for any changes in alkaline phosphatase. The results showed that the positive reaction of the circular muscles at the anterior end of the midgut was slightly reduced. But the enzyme was apparently unchanged in the epithelium of the rectal glands, in the nervous system, and the spiral muscles of some malpighian tubules. In addition there were deposits of phosphate at the distal end of many of the epithelial cells of the large intestine. These occur to some extent in normal *Blattella* and are probably the blue-staining granules reported previously in these cells (Day 1949). In short, the diet containing 10 per cent. *d*-glucoascorbic acid had only a very slight effect on the histochemically detectable alkaline phosphatase.

V. DISCUSSION

(*a*) *Correlation between Histochemical and Chemical Data*

The phosphatases of insects have been studied chemically by Nakamura (1940), Drilhon (1943), and Drilhon and Busnel (1945). The first author found alkaline phosphatase in the silk gland of *Bombyx*, while the later authors found in a number of insects from various orders that the foregut contained a low concentration of alkaline phosphatase and the midgut a stronger concentration, whereas in the hindgut the enzyme was lacking. While they found high concentrations of phosphatase in the malpighian tubules of all species, it was acid rather than alkaline phosphatase and so would not show up in the method used in the present study. The histological results agree with the distribution indicated by chemical methods as far as they go.

(*b*) *Distribution of Enzyme in Tissues of Vertebrates and of Insects*

The majority of vertebrate tissues in one species or another have been found to contain phosphatases (Gomori 1941). They occur in so many locations that their functions must be many and varied. Moog, in her excellent review (1946), has considered phosphatases in relation to calcification, in transport mechanisms, and in relation to growth and differentiation. But even these broad categories do not include the functions of all phosphatases, as for example, the muscle phosphatase of Knoevenagel (1940), that found in the present study, and the phosphatases of the central nervous system (see, for example, Carandante 1941). It will be instructive to compare the distribution in insects with that in vertebrates. So far as calcification is concerned there is no comparison in insects, the place of bony structures being taken by cuticular exoskeleton and apodemes, few of which are associated with alkaline phosphatase. But there are many examples in which the distribution of the enzymes is compatible with a possible

function in transport mechanisms. Bradfield's report (1946) may be considered in this category, and so may the midgut, malpighian tubule, "rectal gland," and male accessory gland phosphatases of this paper.

We have no data on the presence of alkaline phosphatase in insect embryonic tissue, except its observation in the nervous system of *Periplaneta* embryos.

Two interesting points remain to be considered. The first is the wide occurrence of the enzyme in the epithelium of the pharynx. To this organ is usually attributed no function other than the transport of food from the buccal cavity into the crop. Moreover, the epithelium is overlaid by a chitinous intima, sometimes of considerable thickness. When the cytoplasm is continued into this intima as in sense organs, it still gives the positive reaction. The function of the enzyme in this epithelium is an interesting problem and the extent to which this region is capable of absorption requires study. Perhaps the presence of the enzyme can be correlated with the activity of this portion of the gut.

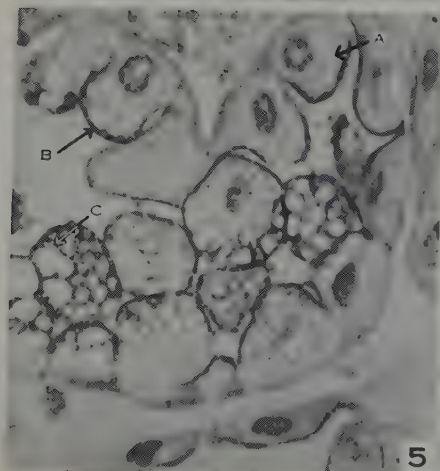
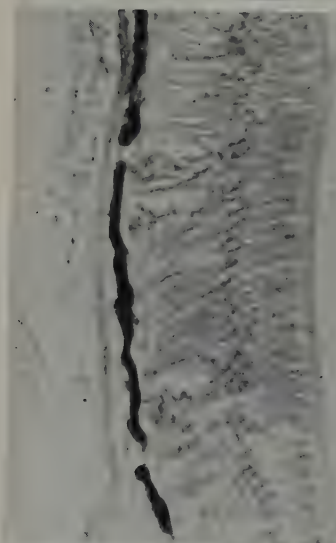
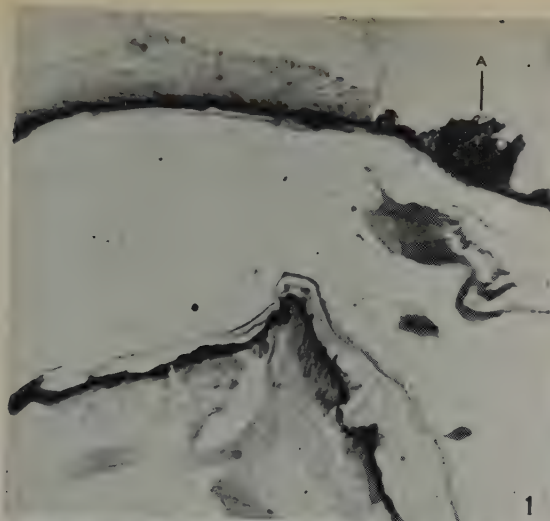
The second point of interest is the degenerating midgut epithelium in the *Lucilia* pupa. The enzyme appears to increase with progressive vacuolization of the cells as though it were playing a part in their destruction. No function for the degenerating midgut cells is known at this stage. In vertebrates the intestinal mucosa is always rich in alkaline phosphatase. The hypothesis has arisen that the enzyme may be involved in the transport of glucose and other molecules through the gut wall. If this is so in vertebrates, some other mechanism must exist in insects, for the enzyme is not conspicuous in the midgut of most species, which region is generally considered to be of considerable importance in absorption. In *Blattella*, when a positive reaction occurs, it is in the golgi zone (cf. Emmel 1945), presumably more concerned with secretion than absorption. The striated border of some tissues is positive, of others negative.

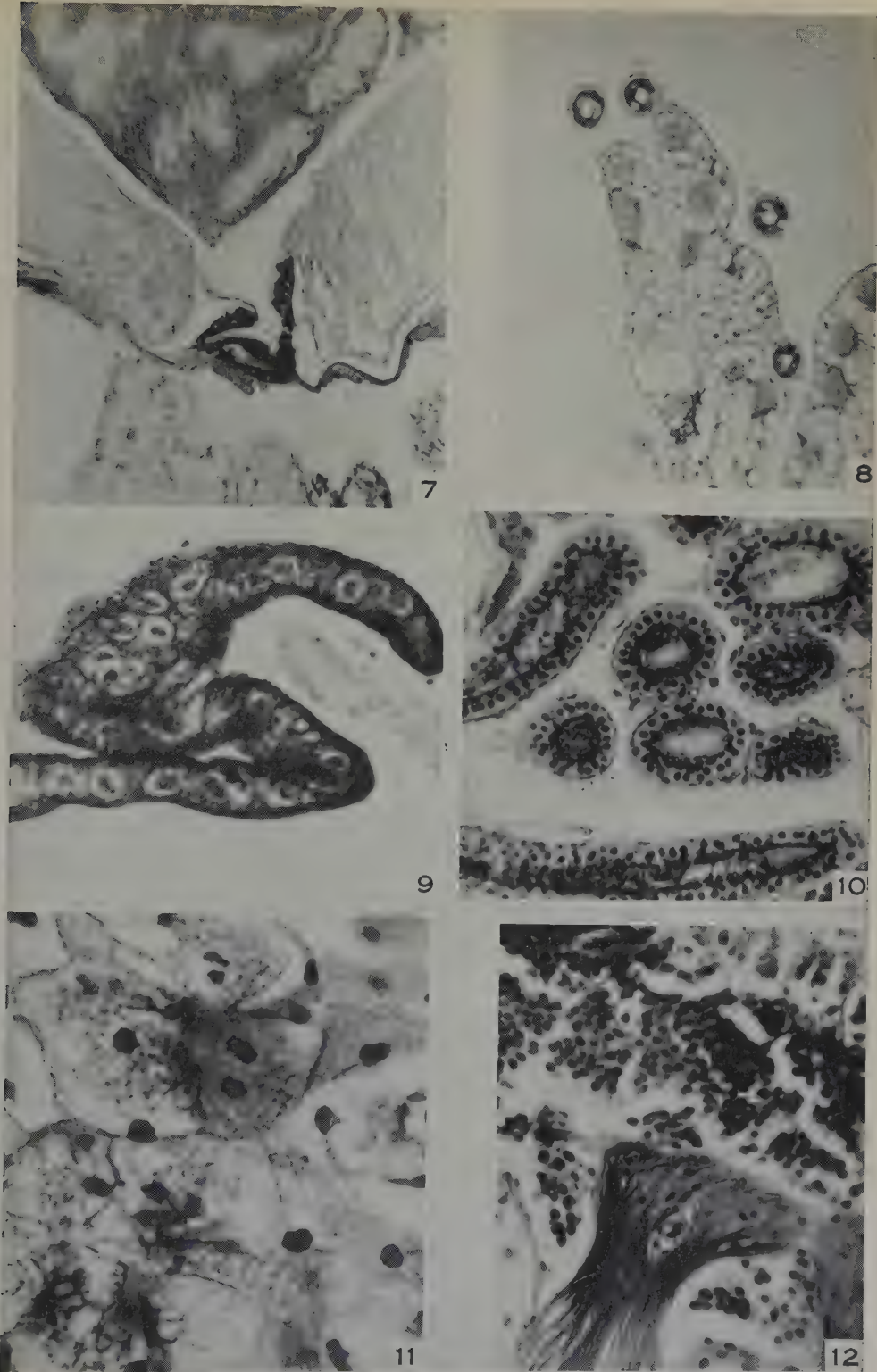
Finally, mention should be made of those tissues in the species examined in which alkaline phosphatase could not be detected despite careful study. These include dermal tissue, pericardial nephrocytes, heart, corpora allata, corpora cardiaca, and imaginal discs (the latter were studied only in *Lucilia*). Two other tissues, fat body and oenocytes, were negative in every species examined except one, and in each exception the tissue was strongly positive. A great deal more will have to be learned of the functions of these organs before an explanation can be advanced.

(c) *Distribution of Inorganic Phosphate*

Inorganic phosphate deposits are readily observed both in slides stained for alkaline phosphatase and in the control slides. It is interesting to note that considerable deposits are found in some insect tissues. The accumulation in the lumen of the malpighian tubules of *Lucilia* and in the cytoplasm of the malpighian tubules of a number of species, has already been mentioned.

The hindgut of *Blattella* normally contains a little phosphate (Plate 3, Fig. 18), but the deposits increase when the insects are fed *d*-glucoascorbic acid or inorganic phosphate. In *Ctenolepisma*, however, the hindgut normally contains





DAY.— THE DISTRIBUTION OF ALKALINE PHOSPHATASE IN INSECTS



DAY.— THE DISTRIBUTION OF ALKALINE PHOSPHATASE IN INSECTS

granules of phosphate. Hoskins and Harrison (1934) recognized that the contents of the honey bee midgut were unusual in containing a large amount of phosphate (0.046M), but found that the excreta contained very little. They considered that absorption occurred in the hindgut. However, the histochemical method demonstrates very considerable deposits in the cells of the midgut (Plate 3, Fig. 17) where it occurs particularly in the apical regions of the epithelial cells at the apex of the folds. Koehler (1920) considered that the midgut cells contained "lime." It is interesting that the granules normally present in *Ctenolepisma*, *Apis*, and those which appeared in the hindgut of *Blattella* are all very similar in size, but those found in the *Lucilia* malpighian tubules are much larger.

VI. ACKNOWLEDGMENTS

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EXPLANATION OF PLATES 1-3

PLATE 1

- A Zeiss IBSO attachment was used for the photomicrographs taken under oil immersion. For lower powers a sliding copying attachment for the Leica camera was employed. Section 10 microns, stained for alkaline phosphatase by Gomori technique. Various magnifications.
- Fig. 1.—*Ctenolepisma*, L.S. pharynx. Buccal cavity to the right. Note entire pharyngeal epithelium is positive, especially sense organ (A) with positive cytoplasm extending through chitinous intima, on dorsal side at the buccal end. x 160.
- Fig. 2.—*Locusta*, L.S. pharynx. Positive epithelium beneath thick chitinous intima. x 220.
- Fig. 3.—*Periplaneta*, L.S. midgut. Epithelium, longitudinal muscles, trachea negative. Only circular muscles are positive. x 380.
- Fig. 4.—*Lucilia* larva, midgut epithelium. Note positive region of cells towards lumen and positive polytene chromosomes. x 220.
- Fig. 5.—*Lucilia* pupa, midgut cells showing increase in phosphatase activity with increasing vacuolization (degeneration) of midgut cells (A to C). x 140.
- Fig. 6.—*Lucilia* adult, T.S. abdomen. Rectal papillae show phosphatase on inner and outer cell borders. Malpighian tubules and male accessory glands also positive. x 140.

PLATE 2

Details of photomicrographs as in Plate 1.

- Fig. 7.—*Blattella*, T.S. rectum. Only cells giving positive reaction are those of the thin epithelium between the rectal pads. x 140.
- Fig. 8.—*Periplaneta*, fat body. Positive regions mainly contiguous with bacteroid containing cells. Malpighian tubules contain granules of phosphate. x 140.
- Fig. 9.—*Locusta*, fat body. The cytoplasm of the fat body is positive, the nuclei are strongly positive. x 380.
- Fig. 10.—*Blattella*, male accessory glands. Nuclei strongly positive, distal cell borders positive. Surrounding fat body negative. x 140.

Fig. 11.—*Blattella*, salivary gland. Nuclei strongly positive, cytoplasm around central ductules positive. x 620.

Fig. 12.—*Locusta*, brain optic lobes. Nuclei positive, many nerve fibres positive, but not in all regions of the brain. x 380.

PLATE 3

Details of photomicrographs as in Plate 1.

Fig. 13.—*Blattella*, T.S. hindgut — large intestine. Positive zone on periphery of some epithelial cells only. Nuclei in this region also positive. x 380.

Fig. 14.—*Locusta*, L.S. rectum showing unusual appearance of positive tracheal end cells and intracellular tracheoles. x 140.

Fig. 15.—*Blattella*, L.S. portion of malpighian tubule. Spiral muscle in section positive (A). The cytoplasm contains alkaline phosphatase and also granules of inorganic phosphate. x 380.

Fig. 16.—*Pieris*, oenocytes, cytoplasm intensely positive. Nucleus also positive, and nuclei of surrounding fat body similarly positive. x 620.

Fig. 17.—*Apis*, T.S. midgut showing inorganic phosphate granules especially in apical regions of epithelium. x 380.

Fig. 18.—*Blattella*, L.S. hindgut — small intestine, inorganic phosphate granules in distal regions of epithelium. x 380.

STUDIES OF COMPARATIVE FLEECE GROWTH IN SHEEP

I. THE QUANTITATIVE NATURE OF INHERENT DIFFERENCES IN WOOL-GROWTH RATE

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[Manuscript received November 5, 1948]

Summary

Since wool-growth rate is strongly influenced by the plane of nutrition, any measure of inherent wool-producing capacity in the sheep must take this into account, and the relation of these three factors to each other must be known. On general grounds, the relation of wool-growth rate to nutrient intake seems likely to follow the familiar law of diminishing returns which introduces the concept that for each sheep there may be an asymptotic value of wool-growth rate characteristic of the individual. From this and other considerations, it was postulated that these relationships could be described by an equation of the form

$$y/A = 1 - e^{-k'(x-x_0)/A},$$

where y = wool-growth rate, x = nutrient intake rate at or above maintenance levels, x_0 = the nutrient intake rate for which $y = 0$, A = the asymptotic value of y , and k' = a constant dependent on the nutritive qualities of the diet employed.

In this paper, an experiment is described which was designed primarily to test the validity of this generalization by comparing estimates of the parameter k' for sheep types contrasting widely in wool-producing capacity. Twelve Camden Park Merinos and twelve Corriedales individually fed were each divided into three groups of four sheep, one group being kept at a uniform intermediate plane of nutrition throughout, one group being transferred in four stages of six weeks duration from a high to a low plane, and one group transferred in four similar stages from a low to a high plane of nutrition. Equilibration periods of not less than 12 weeks were spent at the highest and lowest planes of nutrition before and after the reciprocal transfers were made. The diet used was of constant composition throughout and consisted of lucerne hay chaff 60 parts, rolled wheat 20 parts, and linseed meal 20 parts. No supplement of any sort was provided.

Data from the groups maintained at a uniform plane of nutrition revealed that factors other than the plane of nutrition were causing significant fluctuations in wool-growth rate which required assessment before the main issues could be examined. Wool-growth rate was found to have a significant positive association with the environmental air temperature. For the Uniform groups wool-growth rate increased by 0.048 ± 0.011 g. and 0.082 ± 0.021 g. per sheep per day per °F. in the Camden Park Merino and Corriedale respectively. Analyses on the data for the individual sheep showed significant differences between the regression coefficients for the individual Corriedale sheep. Evidence from the other groups suggested that the change in wool-growth rate (per °F.) increases with the plane of nutrition. It was postulated that the temperature effect was mainly due to cutaneous blood circulation changes which form part of the normal heat regulating response of the body.

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Another significant but inverse association revealed by the data of the Uniform groups occurred between fibre thickness and the amount of fleece carried by the sheep. For each growth increase of 1 lb. in fleece weight there was a mean reduction in fibre cross-sectional area of $6.72\mu^2$ in the Camden Park Merinos and $7.30\mu^2$ in the Corriedales. This phenomenon was ascribed to the effect of fleece covering on the moisture content of the skin and thereby on the tissue hydrostatic pressure, an increase in pressure causing compression of the follicle and the formation of a longer and thinner fibre with no change in the weight of wool produced per unit time. Considerations of tissue hydrostatic pressure were also invoked to explain why the effect of temperature on wool-growth rate was almost wholly brought about by an effect on length-growth rate in contrast to the effect of the plane of nutrition on both fibre thickness and length-growth rate.

The confounding effect of atmospheric temperature on the data for the sheep taken from a high to a low and from a low to a high plane of nutrition prevented a rigorous estimate of all the parameters in the above equation. However, the value of x_0 appeared to be close to zero, and by assuming that it was in fact zero, the other parameters could be estimated from the group mean data for the three groups. A small inaccuracy in the value of x_0 does not seriously bias the estimates of k' and A for the two breeds. The value of k' for the Camden Park Merinos was estimated to be 0.487 and for the Corriedales 0.422. Although the data do not provide standard errors for these estimates, the difference between the two breeds was not regarded as sufficient to dispute the main hypothesis embodied in the equation. The asymptotic value of wool-growth rate was found to be 5.3 g. and 21.9 g. per day respectively for the Camden Park Merino and Corriedale. Using the mean value of k' , 0.454, and putting $x_0 = 0$, a nomograph was constructed showing the family of curves corresponding to intervals of 5 g. per day in the value of A .

It was concluded that although the inherent wool-producing capacity of a sheep is difficult to assess in terms required by the equation, except under laboratory conditions, due weight must be accorded the relationships expressed, especially if Mendelian studies on wool growth in the sheep are to prosper. The experiment has also emphasized that studies on the physiology of heat regulation in the sheep, and especially the role of the skin, must be integrated with nutritional research if a complete understanding of fleece-growth phenomena is to be attained for application in experimental genetics.

I. INTRODUCTION

It is common experience that wool-growth rate is influenced by the environmental history of the sheep, particularly by the plane of nutrition. Consequently, any expression of the inherent productivity of sheep in terms of wool weight produced per unit time is indefinite unless the plane of nutrition is specified. Mere specification of the plane of nutrition, however, will not suffice unless the relation between wool-growth rate, plane of nutrition, and the maximum wool-producing capacity of the sheep is known. The purpose of this paper is to examine this relationship.

On general considerations it seemed likely that the relation of wool-growth rate to the nutrient intake would follow the familiar law of diminishing returns. The sheep as a living body is more adaptable than a mechanical engine and increases structurally and functionally in response to an increase in nutrient

intake, but does not do so *proportionately* and eventually reaches a maximum capacity for wool-growth rate. This point may or may not be reached before the maximum food capacity of the sheep. We thus have the concept of an asymptotic value of wool-growth rate characteristic of the individual.

The relationship may be further defined by restricting it to nutrient intakes above maintenance, unless, below maintenance, the nutrient intake value be adjusted to include nutrients supplied by the catabolism of body tissues. When so adjusted a nutrient intake below maintenance cannot be achieved in practice. However, for an *imaginary* nutrient intake of x_0 , where $x_0 \geq 0$, we may postulate that the corresponding wool-growth rate becomes zero. The value of x_0 may be taken as indicating the threshold above which wool-growth rate commences. It is impossible, from energetic considerations, to imagine a positive value of wool-growth rate corresponding to an imaginary adjusted nutrient intake of zero. Mathematically, the above conditions are met by the equation

$$y = A - Ae^{-k(x - x_0)} \dots \dots \dots (1)$$

where y = the wool-growth rate, x = the nutrient intake rate, x_0 = the nutrient intake rate for which $y = 0$, A = the asymptotic value of y , and k = a constant dependent on the particular foods used and representing the percentage decline in wool-growth rate increment per unit increase in nutrient intake.

This form of equation has been used to relate net energy to gross energy intake by Wiegner and Ghoneim (1930) in rabbits, and by Brody and Proctor (1933) and Brody (1945) in steers. It has been used to relate milk-yield to nutrient intake in dairy cattle by Jensen *et al.* (1942). Brody (*loc. cit.*) has also used the equation to describe the self-limiting phase of growth of populations and single organisms. To our knowledge the equation has not previously been used to describe the relation of wool production to nutrient intake in sheep.

Differentiating equation (1) we obtain

$$dy/dx = k(A - y),$$

which provides the concept that the conversion efficiency of an additional increment of nutrient intake is proportional to the degree of saturation of the physiological system involved in the transformation of the nutrients into wool. This concept formed the basis of a generalization of equation (1) proposed by one of us for the relation of milk-yield to the nutrient intake (Ferguson 1944). If we suppose that the physiological system for the transformation of nutrients to wool remains qualitatively the same for sheep of different productive capacities, equivalent degrees of saturation for such sheep are given by a single value of $(A - y)/A$. Thus we write

$$dy/dx = k'(A - y)/A$$

Integrating

$$y/A = 1 - e^{-k'(x - x_0)/A} \dots \dots \dots (2)$$

In this equation, A , while remaining a constant for a particular sheep, becomes a variable for different sheep. The value of k' remains constant for all sheep but depends on the nutritive qualities of the diet employed.

The validity of the generalization involved in equation (2) may be tested by comparing estimates of the parameter k' for sheep types contrasting widely in wool-producing capacity. The present experiment was set up primarily to make this comparison. The object of the experiment thus stated deals only with the quantitative nature of inherent differences in wool-producing capacity. The problem of the physiological nature of these differences requires further experimental investigation and is only briefly considered in this paper.

The experimental programme thus established provided the opportunity for the collection of data not directly concerned with our present objective. These extraneous data will not be considered here although a complete list of the observations made is included.

II. THE EXPERIMENT

(a) *The Experimental Design*

In attempting to estimate a particular relationship it is important to define the conditions under which the relationship is valid and to provide these conditions in the experiment.

As a primary condition, the plane of nutrition for our present purpose has been taken as the total consumption of nitrogen from a qualitatively suitable ration in which the composition remains unaltered at all levels of feeding. Under such conditions, clearly, the intake of any particular constituent of the ration is proportional to the intake of any other, but nitrogen was chosen as the most logical determinant of the amino acids assimilated, which in turn form the substrate of the keratin laid down in wool growth.

A further condition is that when fleece growth is being measured it should be in equilibrium with the plane of nutrition. Obviously, the period required before such equilibrium is established will depend on the magnitude and direction of the change in plane of nutrition and the physiological status of the animal. Equilibrium on nutrient intakes supplying less than maintenance requirements cannot be maintained once the supply of nutrients from the catabolism of body tissues becomes exhausted. If data obtained on nutrient intakes below maintenance are to be used, it would be necessary to adjust the nitrogen intake for the nitrogen supplied by the catabolism of body tissues. Such an adjustment cannot be made without data on the nitrogen balance of the sheep, so that for the present experiment it was decided to estimate the parameters A , k , and k' , and also x_0 from data obtained at or above maintenance levels. However, the measurement of fleece growth at levels below maintenance was included in the experimental plan.

Next, the sheep used must have reached their mature rate of fleece growth if the effects of differences in the plane of nutrition are not to be unduly confounded with age changes.

A complete test of the hypothesis can only be made by studying a wide range of sheep types, but with our limited facilities, it was necessary to reduce these to two, namely, the Camden Park Merino and the Corriedale. These types were selected since they contrast widely in a great range of characters, particularly in wool-producing capacity.

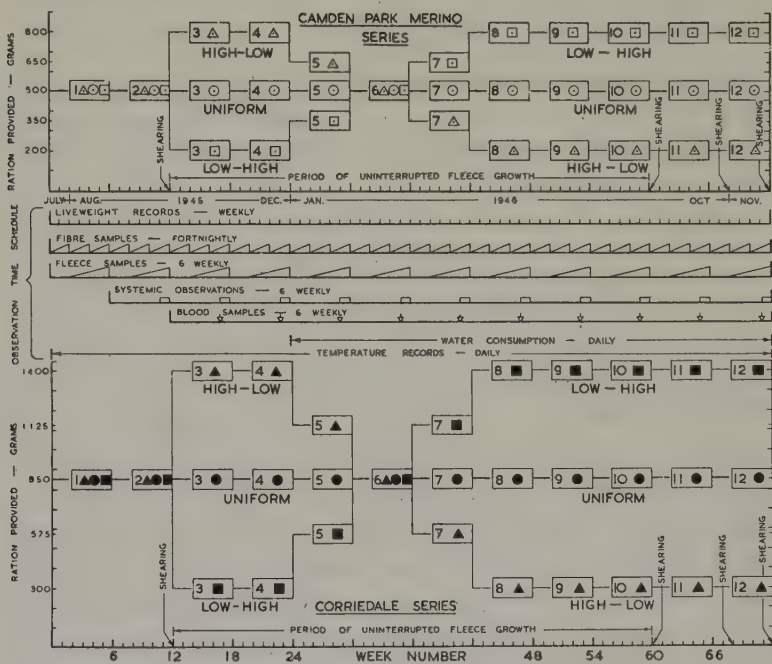


Fig. 1.—The plan and observation time schedule of the experiment.

The plan of the experiment is given in Figure 1. Each sheep type was represented by three groups of four sheep. Subject to restriction by liveweight, these sheep were allotted to their respective groups at random. In each series, the groups are hereafter referred to as the High-Low, Uniform, and Low-High groups respectively. The Uniform group was maintained on a constant plane of nutrition throughout to measure the effect on fleece growth of changing atmospheric or other environmental conditions common to all the sheep, as well as to provide some measure of possible time trends due to aging.

The High-Low group after a preliminary period of twelve weeks on the level of the intermediate Uniform group was raised in one step to a high plane of nutrition and brought down in four lesser stages of six weeks each to a low plane. The Low-High group followed the reverse procedure.

The five levels of intake of food per day thus established were, for the Corriedales, 1400 g., 1125 g., 850 g., 575 g., and 300 g. and for the Camden Park Merinos, 800 g., 650 g., 500 g., 350 g., and 200 g. The time intervals between successive observations varied for each character measured and are discussed later.

For about two months prior to the experiment, the sheep were allowed to become accustomed to their pens and during this period were fed *ad libitum* on lucerne chaff. In that time an estimate was made of the food capacity of the sheep so that the several levels of intake could be spaced over the full range of their appetite. Observations made in 1944 by one of us (Carter, unpublished data) indicate that the appetite markedly increases after shearing and then declines steadily until the next shearing. To avoid food residues this factor had also to be considered and the highest levels of intake for each sheep type were fixed somewhat below the estimated *ad libitum* capacity.

(b) *The Experimental Animals*

The Camden Park Merino ewes are direct descendants of the original Macarthur flock established at Camden, New South Wales, during the first decade of the last century. The flock has been maintained as an historic residue by the Trustees of the Estate and entirely closed to the introduction of outside blood for the past sixty or seventy years. This small flock thus constitutes one of the most highly inbred strains of sheep available and is particularly uniform in general type. The Camden Park Merino is not now of any commercial significance in Australian sheep-breeding. There is only one other Australian flock of a similar type in existence, also retained mainly for its historic interest. Both flocks typify the fine-wooled Australian Merino characteristic in this country of the period before 1870.

The sheep are small by modern standards although well-knit and vigorous, are almost entirely free from skin wrinkles and folds, and carry a fine fleece of high wax content. The skin supports a fibre population of moderate density. Their wool-productive capacity is low but not greatly below their modern equivalent. Initially they are nervous under restraint and may require some time to settle down to confined pen conditions. This is not regarded as an inherent characteristic so much as due to the relatively infrequent handling on their home pastures.

The Corriedale ewes were obtained from a stud flock at Trangie, New South Wales. By contrast, they are large, carry a strong fleece, low in wax content but high in suint. The fibre-population density of the skin is relatively low. These sheep have a moderate to high wool-producing capacity. They are, like the Camden Park Merino, relatively free from skin folds and wrinkles and this reduces the error of measurement of fleece-growth rate. They proved to have a more placid temperament than the Camden ewes under close confinement.

When the experiment started, the age of the Camden Park Merinos was about 23 months and of the Corriedales about 26 months.

(c) *The Diet*

The selection of a diet acceptable to sheep during long periods of close confinement and suitable for studies of this kind is difficult. In this experiment,

it was desirable to compound a food mixture with a sufficient concentration of nitrogen in available form to permit high intakes within the dry matter capacity of the sheep.

The fodder WB.3 used in the experiment, therefore, consisted of a loose mixture of good quality sun-cured lucerne hay chaff 60 parts, rolled wheat 20 parts, and linseed meal 20 parts. Apart from free access to clean tap water, the diet consisted of this mixture only, no mineral or other supplement being provided at any time during the course of the experiment. The full supply of fodder necessary for the whole experiment was mixed as one large batch to ensure

TABLE 1
SUMMARY OF THE PRINCIPAL DIETARY CONSTITUENTS PROVIDED AT EACH LEVEL
OF FEEDING

Plane of Feeding	Camden Park Merino Series						Corriedale Series					
	Amount provided per Sheep per 24 Hours						Amount provided per Sheep per 24 Hours					
	Dry Matter (g.)	Total Energy (kg. cal.)	Total Nitrogen (g.)	Total CaO (g.)	Total P ₂ O ₅ (g.)	Total Copper (mg.)	Dry Matter (g.)	Total Energy (kg. cal.)	Total Nitrogen (g.)	Total CaO (g.)	Total P ₂ O ₅ (g.)	Total Copper (mg.)
1 (High)	712	3240	24.6	10.1	6.3	8.0	1248	5680	43.2	17.6	11.1	14.0
2	580	2630	20.1	8.2	5.2	6.5	1000	4960	34.6	14.1	8.9	11.3
3 (Uniform)	445	2025	15.4	6.3	4.0	5.0	757	3440	26.2	10.7	6.7	8.5
4	312	1420	10.8	4.4	2.8	3.5	512	2330	17.7	7.2	4.6	5.8
5 (Low)	178	810	6.0	2.5	1.6	2.0	267	1215	9.3	3.8	2.4	3.0

reasonable constancy of composition. It should be noted, however, that this intention was not entirely fulfilled since at the end of Period 9 the stored fodder was accidentally damaged by water, and for the remaining periods, a new batch of similar ingredients was used. Throughout the experiment, samples were drawn daily from each individual ration, and bulked for each experimental period of six weeks into one lot which was subsampled for analysis. From these analyses the following mean values were obtained on a dry weight basis: crude protein ($N \times 6.25$) 21.6 per cent.; crude fat (ether extract) 5.4 per cent.; crude fibre 19.1 per cent; N-free extract 45.6 per cent.; ash 8.2 per cent. The diet also contained CaO 1.41 per cent.; P₂O₅ 0.89 per cent.; copper 11.3 μ g./g. The combustible energy of the mixture was 4.56 kg.cal./g. dry weight, whereas the mean moisture content as fed to the sheep was 10.9 per cent. The amounts of the principal dietary constituents provided at each of the given levels of feeding are summarized in Table 1. The nitrogen intake was calculated for each period from the analytical value obtained on the sample for that period (Table 2).

Analyses of duplicate subsamples of the bulked food samples in the first 7 periods gave ± 0.09 as the standard error of the individual values in Table 2. However, the error of the bulk sampling is unknown.

TABLE 2
NITROGEN CONTENT OF THE EXPERIMENTAL DIET

Period No.	1	2	3	4	5	6	7	8	9	10	11	12
Nitrogen												
Per Cent.	3.38	3.36	3.36	3.38	3.54	3.66	3.70	3.46	3.43	3.30	3.27	3.23
Dry Weight												

A digestibility trial with the mixture was carried out during Period 12. Six sheep were used representing each of the six groups in the experiment. The object of the trial was to obtain an average value for the apparent digestibility of the experimental diet. The sheep were confined in metabolism cages similar to those described by Marston (1935). The fodder and faeces were weighed and sampled over a period of 7 days. Subsamples of fodder and faeces were analysed for combustible energy and nitrogen content. The results are given in Table 3.

TABLE 3
SUMMARY OF DIGESTIBILITY TRIAL WITH THE FODDER WB.3

Breed	Sheep No.	Ration Fed (g. per 24 hr.)	Ration Consumed (g. per 24 hr.)	Nitrogen (g. per 24 hr.)		Digestible Nitrogen (%)	Energy (kg. cal. per 24 hr.)		Digestible Energy (%)
				Intake	Faeces		Intake	Faeces	
Corriedale	244	1400	1225	32.19	7.94	75.5	5008	1384	72.4
	247	850	850	22.80	4.38	80.8	3455	842	75.6
	246	300	300	8.06	1.38	82.9	1221	244	80.0
Camden	236	800	457	11.87	2.52	78.7	1770	464	73.8
Park	228	500	500	13.41	2.77	79.4	2031	510	74.9
Merino	227	450	450	12.07	2.52	79.1	1830	494	73.0

Unfortunately the trial was marred by the necessity for increasing the intake of sheep No. 227 shortly before the trial in order to keep her alive and by the refusal of sheep No. 244 and No. 236 to consume their whole ration towards the end of the trial. Circumstances made it impossible to continue the trial until perfect behaviour of all the sheep was obtained. Notwithstanding this, the digestibility coefficients for the several sheep show reasonable agreement. The values do not suggest any radical difference in the digestive abilities of the two breeds.

(d) *Observations and Methods*

(i) *General Husbandry*.—The animals were confined individually throughout the course of the experiment each in a small single pen measuring 5 feet by 4 feet, within a well-constructed sheep house, with concrete flooring, single course brick walls and fibrocement roofing provided with sky-lights. The sheep were thus entirely protected from direct sunlight, rain, and to a considerable extent from turbulent air movement which seldom exceeded 60 feet per minute within the pens. Under these conditions, it was not considered necessary to rug the sheep, though rugging was omitted for the further reason that it would interfere too greatly with the normal production of the skin secretions by altering the fleece micro-climate. An additional reason for excluding rugs was that they absorb these secretions to a degree sufficient to vitiate the results of the periodic chemical fractionations made.

All pens were cleaned daily, except at week-ends, between 8.0 a.m. and 9.0 a.m. by sweeping and liberal hosing, each animal being removed from its pen during the process. Movable wooden gratings were provided over half the floor of each pen, and this provision, together with the regular cleaning, tended to minimize soiling of the fleece and any risk of re-infestation with internal helminths. Food and water were provided in specially made galvanized iron troughs clipped to the iron railings at the front of each pen. Food was offered daily between 9.0 a.m. and 9.30 a.m. and the residues, if any, were collected and weighed. From Period 5 onwards, the daily water consumption was measured by filling each trough to a fixed mark while the sheep were consuming the new day's food ration. The daily food rations and residues were weighed to the nearest 5 g. and the daily water consumption measured to the nearest 100 ml.

The only occasions on which the animals were removed from their pens for any appreciable time were weekly for weighing, six-weekly for fleece sampling, and on three occasions to be photographed. All other manipulations (e.g. fibre clippings, skin biopsies, bleeding, clinical observations, etc.) were done with the animals standing quietly in their pens.

Periodic faecal examinations were made to estimate the degree and nature of helminth infestation. Phenothiazine was administered early in the experiment and thereafter the degree of infestation remained at an insignificant level.

(ii) *Fleece-Growth Rate*.—Fleece-growth rate was measured over the last four weeks of each six-weekly period by clipping the fleece from defined areas (10 cm. by 10 cm. approximately) on the midside of each sheep. The areas were delineated by tattooing. The sizes of the sample areas were measured at the conclusion of each period, first with the sheep in the lateral recumbent position on the table used for sampling, and again with the sheep standing normally in the pen. The size of each patch was determined by measuring the four sides of the square with a flexible celluloid rule and multiplying the mean of the dorsal and ventral by the mean of the anterior and posterior margins.

The fleece clipped from each sample area was weighed and the main components, viz. clean dry wool, wax, and suint, were determined. The "wax" fraction was separated by soxhlet extraction with carbon tetrachloride. The "suint" fraction was the cold water soluble material extracted after treatment with carbon tetrachloride. In the course of analysis the quantities of moisture and dirt in the samples were also obtained.

TABLE 4
RELATION OF FLEECE PRODUCTION ON MIDSIDE TO TOTAL FLEECE PRODUCTION
(CAMDEN PARK MERINO SERIES)

Group	Sheep No.	Skin Area (Periods 1 and 2)	Ratio of Value for the Whole Sheep to Value for the Sample Area				Ratio of Production per Unit Area over the Whole Sheep to Production per Unit Area of the Sample			
			Wool	Wax	Suint	Total Skin Products	Wool	Wax	Suint	Total Skin Products
High-Low	227	78.2	57.3	70.3	100.8	64.7	0.733	0.899	1.289	0.826
	232	83.8	63.5*	83.3*	88.1*	71.3*	**	**	**	**
	234	84.7	64.2*	74.2*	89.1*	72.1*	**	**	**	**
	241	80.0	60.7*	79.5*	84.1*	68.1*	**	**	**	**
	Mean	81.7	61.4	76.8	90.5	69.0	0.733	0.899	1.289	0.826
Uniform	225	80.1	59.6	103.6	100.4	72.9	0.744	1.294	1.253	0.910
	226	87.7	66.5*	87.2*	92.2*	74.6*	**	**	**	**
	228	78.2	58.4	72.9	92.4	64.9	0.747	0.932	1.181	0.830
	240	88.2	63.1	52.8	85.2	73.8	0.715	1.064	0.965	0.837
	Mean	83.6	61.9	79.1	92.5	71.6	0.735	1.097	1.133	0.859
Low-High	229	91.4	67.7	79.6	102.3	74.2	0.740	0.870	1.120	0.813
	233	91.6	80.2	107.6	92.9	88.5	0.876	1.174	1.013	0.964
	235	78.5	59.1	59.3	67.0	59.9	0.753	0.755	0.854	0.763
	236	79.9	62.1	98.0	72.7	71.9	0.777	1.227	0.910	0.901
	Mean	85.4	67.3	86.1	83.7	73.6	0.786	1.006	0.974	0.860
Grand Mean		83.5	63.5	80.7	88.9	71.4	0.761	1.027	1.073	0.856

* Estimated value. ** Sheep dead at time of estimation.

The relation of the sample fleece production to the total fleece production was estimated in the last period of the experiment (Period 12) by clipping the entire sheep in addition to the midside areas. A sample of the total fleece was analysed in the usual manner. In the same period, the surface areas of the surviving sheep were measured by means of the surface integrator described by Elting and Brody (1926).

Tables 4 and 5 show the ratios of total to sample fleece growth and total area to sample area for each sheep. The area ratios are those calculated for

Periods 1 and 2.* The ratios of fleece growth per unit area on the midside to fleece growth per unit area over the whole body are also shown. The differences between the groups are not significant and it may be concluded that the ratios were little affected by the plane of nutrition. Consequently, the ratios of total to sample fleece growth were used to estimate the total fleece growth in each period merely by multiplying the sample values of the fleece constituents by a factor constant for each sheep.

Theoretical reasons are given later to support the possibility that the ratio of total to sample fleece growth may have been influenced by the atmospheric temperature. It should be remembered, therefore, that the analysis of the influence of various factors other than the plane of nutrition on fleece growth applies strictly to fleece growth on the tattooed areas and that the proportionality between fleece-growth rate on the tattooed and total areas is established only for changes in fleece-growth rate due to changes in the nutrient intake.

The asterisks in Tables 4 and 5 denote estimated values of the ratios of total to sample wool, wax, and suint production for sheep which died before the end of Period 12.

(iii) *Fibre Thickness*.—A clipping of short lengths of fibre was taken every 14 days from a fixed location about 5 cm. anterior to the fleece sample area on the right side. This was done by first clipping the site with coarse cutter-heads on the electric clippers and then with fine cutter-heads. The fibre fragments obtained in this way were about 1 mm. in length and were formed in the follicles some days previously, the exact time being different for each sheep and depending on the rate of fibre-length growth. The fragments were mounted on a slide and the diameters of the projected images of a hundred fibres measured at a magnification of $\times 500$.

* The values were calculated by dividing estimates of the ratio of total to sample surface area for the missing sheep by the average value of the ratio of total to sample fleece growth per unit area. This procedure takes into account the differences between sheep in the ratio of total to sample area which result from individual differences in the size of area tattooed and from differences in body surface area, but assumes that the sheep did not differ from the breed average in the ratio of total to sample fleece growth per unit area. Estimates of the surface area of sheep in each period were made from equations relating surface area to liveweight, chest girth, and chest depth fitted to the data of Period 12. The equations were

$$\text{Camden Park Merino} \dots S/W^{\frac{1}{3}} = 0.007 + 0.191 D/G$$

$$\text{Corriedale} \dots S/W^{\frac{1}{3}} = 0.054 + 0.393 D/G$$

where S = surface area in square metres, W = liveweight in kilograms, D = chest depth in any units, G = chest girth in same units as chest depth. The ratio of total to sample area for each sheep did not appear to be influenced by the treatment differences and tended to remain fairly constant throughout the experiment. However, to avoid possible treatment effects, the mean values of the area ratios in Periods 1 and 2 were used in estimating the ratio of total to sample fleece growth for the sheep missing in Period 12.

For certain of the following analyses, the square of the mean fibre diameter multiplied by $\pi/4$ to give an estimate of the mean fibre cross-sectional area has been used, although it was realized that the shape of the cross section is seldom truly circular.

TABLE 5
RELATION OF FLEECE PRODUCTION ON MIDSIDE TO TOTAL FLEECE PRODUCTION
(CORRIEDALE SERIES)

Group	Sheep No.	Skin Area (Periods 1 and 2)	Ratio of Value for the Whole Sheep to Value for the Sample Area				Ratio of Production per Unit Area over the Whole Sheep to Production per Unit Area of the Sample			
			Wool	Wax	Suint	Total Skin Products	Wool	Wax	Suint	Total Skin Products
High-Low	246	117.0	100.6	132.3	129.3	113.2	0.860	1.132	1.105	0.968
	250	102.2	82.7	107.8	127.6	91.4	0.809	1.055	1.250	0.894
	251	99.1	81.3*	99.6*	111.3*	86.2*	**	**	**	**
	257	87.5	99.5	120.0	87.6	104.7	1.138	1.370	1.005	1.196
	Mean	101.4	91.0	114.9	114.0	98.9	0.936	1.186	1.120	1.019
Uniform	243	97.8	80.6	95.7	113.3	83.8	0.824	0.978	1.159	0.857
	245	95.3	83.7	78.9	120.9	84.8	0.877	0.827	1.269	0.890
	247	112.8	78.0	105.2	110.0	83.7	0.692	0.932	0.975	0.742
	253	117.4	96.3	127.1	167.4	103.3	0.820	1.082	1.426	0.880
	Mean	105.8	84.6	101.7	127.9	88.90	0.803	0.955	1.207	0.842
Low-High	242	95.4	78.2*	95.9*	107.1*	82.9*	**	**	**	**
	244	104.0	83.1	116.4	123.0	89.5	0.799	1.120	1.183	0.861
	248	114.2	80.3	99.4	89.7	82.9	0.703	0.870	0.785	0.726
	252	117.0	95.0	105.0	163.0	97.6	0.812	0.898	1.397	0.835
	Mean	107.7	84.2	104.2	120.8	88.2	0.771	0.963	1.122	0.807
Grand Mean		105.0	86.6	106.9	120.9	92.00	0.833	1.026	1.155	0.885

* Estimated value. ** Sheep dead at time of estimation.

(iv) *Fibre-Population Density*.—The fibre-population density was estimated by counting the number of fibres in several projections each of 1 sq. mm. of a histological section of the skin. Circular pieces of skin, 1 cm. in diameter, were taken from randomized positions on the dorsal region of each sheep by means of a special skin trephine. The technique described by Carter (1939) was used in the preparation of histological sections. The degree of contraction during preparation of the section, and the resulting effect on density, was determined by estimating the mean diameter of the mounted section and calculating its area. The estimate of mean follicle population density was corrected accordingly.

(v) *Fibre Length*.—A clipping of fibres immediately posterior to each mid-side sample taken at the end of every period provided material for the measurement of fibre-length growth. These measurements have not yet been carried out

but a provisional estimate of length was made for each sheep in each period based on the wool weight, fibre diameter, and follicle population of the tattooed area. The values of wool weight and fibre diameter for the individual periods were used to make this estimate but the follicle population per tattooed area was found to be relatively constant and the mean value for Periods 1-9 was used for each sheep. The fibre-population of the tattooed patch was determined from the fibre-population density, and the area of the tattooed patch measured in the standing position. For some regression analyses not used in this paper, however, an estimate of length was made based on weight of wool per tattooed area and fibre diameter in conjunction with the few available direct measurements of fibre length.

(vi) *Body Weight*.—The sheep were weighed at weekly intervals throughout the experiment at the same hour on each occasion.

(vii) *Body Conformation*.—Several linear dimensions were recorded at the end of each period. These were:

- (1) Standing height at withers.
- (2) Width of chest immediately posterior to the elbows.
- (3) Depth of chest immediately posterior to the elbows.
- (4) Chest girth immediately posterior to the elbows.
- (5) Length from the point of the shoulder to the tuber coxae.
- (6) Width between the tubera coxae.

(viii) *Systemic Reactions*.—At the end of each period after Period 1, observations were made of respiration rate, heart rate, rectal temperature, and skin surface temperature. Again, these measurements will not be considered in this paper except in so far as they may be used to interpret other results. The observations were taken during the afternoon of five days in the sixth week of each period. Skin surface temperature measurements were taken with a clinical thermometer and therefore must be regarded with caution.

(ix) *Blood Analyses*.—During one day of the fifth week of each period after Period 2, jugular blood samples were taken from each sheep between 9 a.m. and 9.30 a.m. at each collection. Determinations were made of blood sugar, blood non-protein nitrogen, blood ketones, serum protein, serum calcium, serum inorganic phosphorus, and serum magnesium. The results of those analyses are not considered in this paper apart from the association between blood non-protein nitrogen and wool-growth rate.

(x) *Atmospheric Temperature*.—The daily maximum and minimum atmospheric temperatures were recorded throughout the experiment at two places in the sheep house, one in the Camden Park Merino group of pens and one in the Corriedale group. The mean of the maximum and minimum daily values for the latter four weeks of each period was used in relating fleece growth to the atmospheric temperature.

(e) The Course of the Experiment

The experiment commenced on July 16, 1945, and observations continued for 72 weeks until the end of November 1946, although the main requirements of the design had been satisfied at the end of Period 9 (July 28, 1946), i.e. at the end of the 54th week.

Slight modification of the original experimental treatment levels proved necessary in order to keep alive, in the early periods, the sheep on the low plane of nutrition.

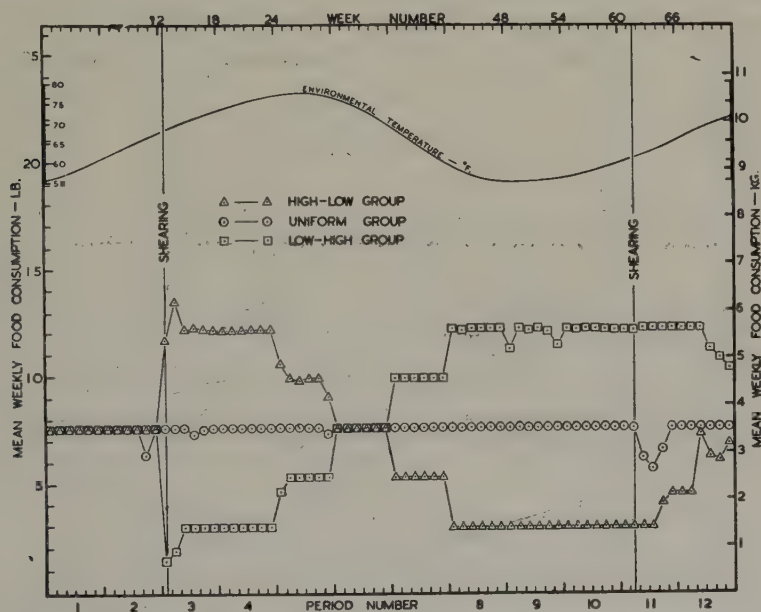


Fig. 2.—Camden Park Merino Series. The mean weekly food consumption per sheep.

During the first fortnight of Period 3, when the groups first received differential treatment, the feed levels for the High-Low groups were 1500 g., and 900 g. per day for the Corriedales and Camden Park Merinos respectively. The corresponding levels for the Low-High groups were 200 g. and 100 g. Before the commencement of the fleece-collection period in Period 3, the ration of the lowest level was increased by 100 g. for each breed and the ration of the highest level decreased by 100 g. The ration of the Uniform groups remained unaltered while the remaining levels of nutrition in the experimental schedule were altered to be half way between the Uniform and the Low, and the Uniform and the High levels respectively. The plan of the experiment in Figure 1 shows the amended levels. Figures 2 and 3 show the actual mean weekly food intake per sheep of each group during the experiment. The different levels received by the High-Low and Low-High groups for 14 days in Period 3, had the desirable effect of bringing the wool-production rate of these groups more rapidly into equilibrium with their respective food intake.

In Periods 11 and 12, the ration of the High-Low Camden Park Merino group was increased when it became apparent that the animals could not exist on the lower ration after they had been shorn at the end of Period 10. Even so, three sheep (Nos. 232, 234, and 241) in this group died from total inanition before the end of the experiment.

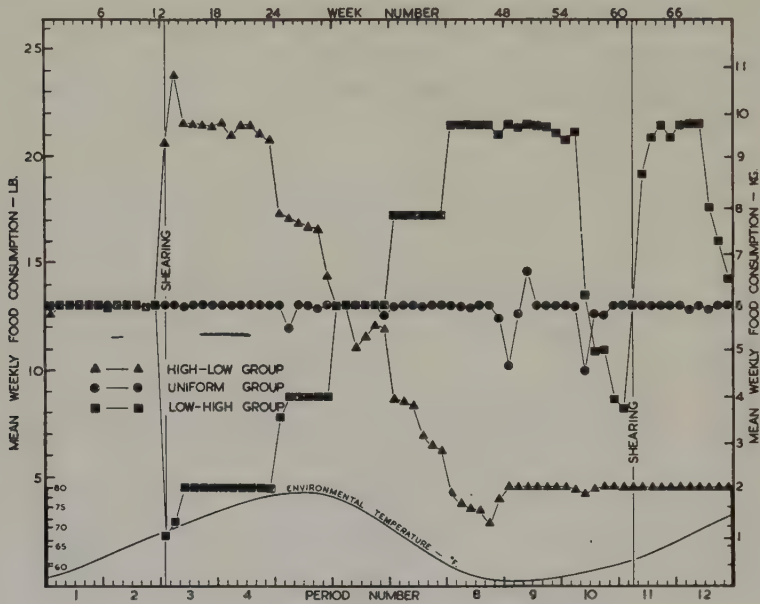


Fig. 3.—Corriedale Series. The mean weekly food consumption per sheep.

Loss of appetite occurred at various times, in three cases leading to death. The extent of inappetence may in part be appreciated from Figures 2 and 3. As mentioned earlier, the remainder of the first food mixture was unavailable in Period 10 and a fresh mixture of the same nominal composition was prepared. The feeding of this mixture was associated with inappetence in several sheep and consequent depression of wool-growth rate. In some cases, wool-growth rate appeared to be affected even though no decrease in food intake occurred. Sheep No. 242 in the Corriedale Low-High group did not recover her appetite and died in Period 11.

Sheep No. 251 of the Corriedale High-Low group showed decreased appetite in Periods 7 and 8 and died in Period 8. Sheep No. 257 in the same group showed decreased appetite in Periods 6 and 7. The observed wool-growth rate of these sheep in the above periods cannot therefore be regarded as being in the same state of equilibrium with the food intake as the wool-growth rate of the other sheep in the same group. Sheep No. 226 of the Uniform Camden Park Merino group showed decreased appetite in Period 11 and died towards the end of that

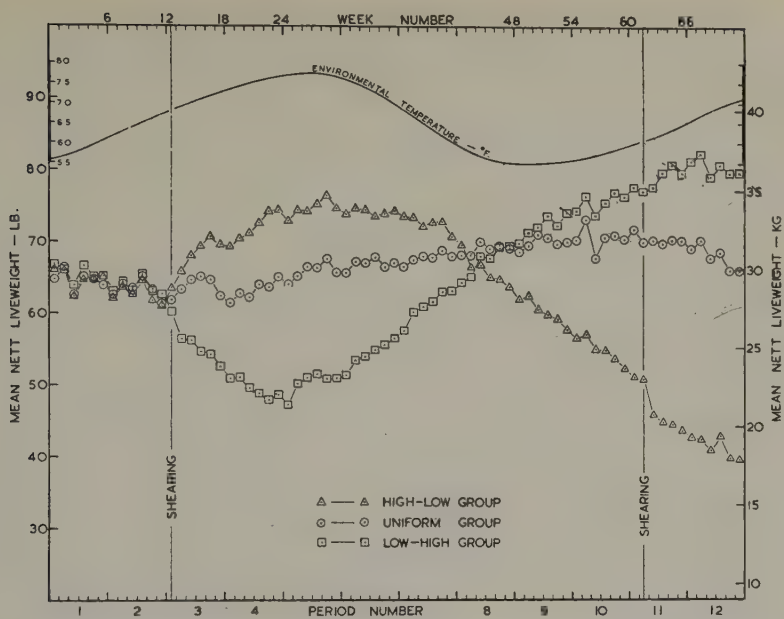


Fig. 4.—Camden Park Merino Series. The mean nett liveweight of the sheep.

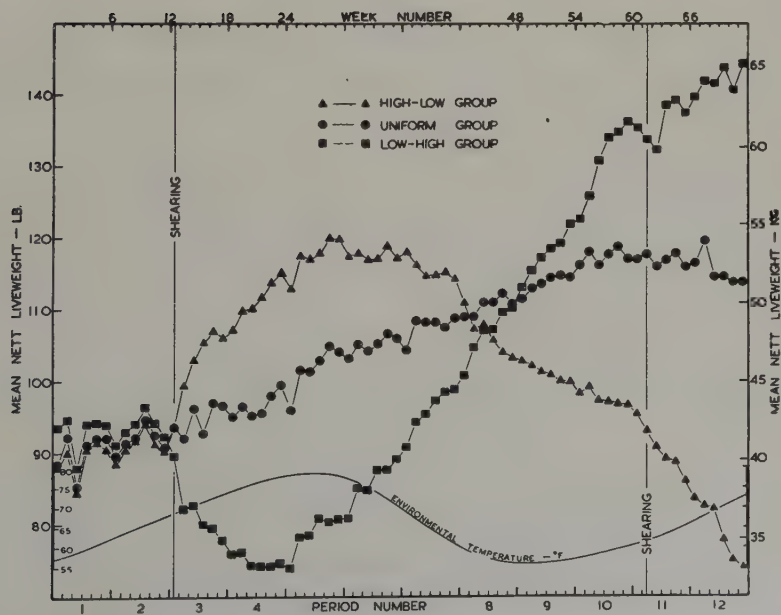


Fig. 5.—Corriedale Series. The mean nett liveweight of the sheep.

period. Transfer of sheep to the metabolism cages for the digestibility trial in Period 12 also caused some depression of appetite. Several substitutions of sheep were made before the trial was commenced.

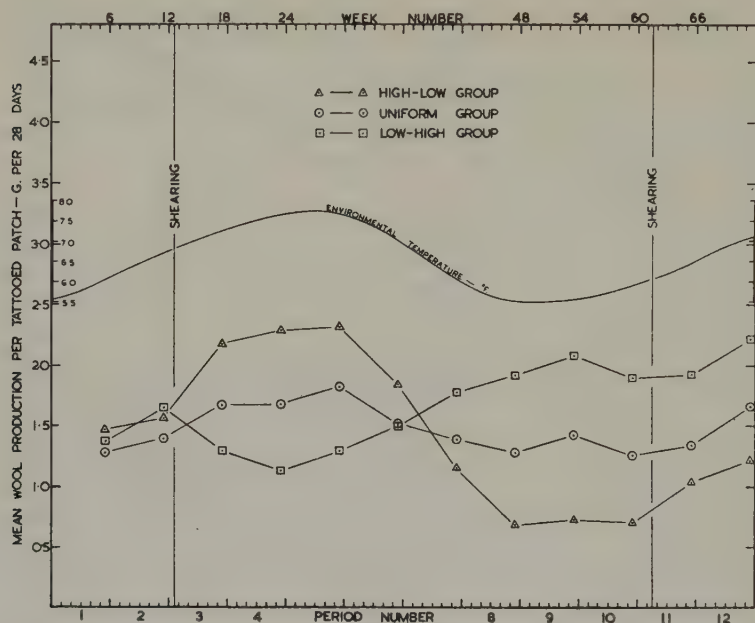


Fig. 6.—Camden Park Merino Series. The mean wool production per tattooed patch, expressed as dry weight per unit time.

The wool-growth rate of most of the Camden Park Merino sheep was low in Period 1 compared with later periods. This may have been due to the change from a ration of lucerne only to the experimental diet at the beginning of Period 1. It is a common experience in experimental work with hand-fed sheep that simple changes in the ration, sometimes, only a change in the batch of food mixture, with no alteration of constituents, can cause a marked disturbance of metabolism. One explanation offered is that a qualitative change in the ration may disturb the normal symbiotic relations of the ruminal flora leading to undesirable fermentations. In the later analyses of the relation of wool-growth rate, plane of nutrition and wool-producing capacity of the sheep, the Period 2 values of wool-growth rate have been used as an indication of the productive capacity of the sheep rather than the mean of the Period 1 and 2 values.

Most of the serious disturbances of the experimental programme occurred in the last three periods. They did not affect the essential aims of the main experiment which is to be regarded as completed at the end of Period 9.

Since helminthiasis, particularly trichostrongylosis, can appreciably alter wool-growth rate even with subclinical infestations (Carter, Franklin, and Gordon 1946) care was taken to see that this factor was excluded. Although all

sheep entered the experiment with a slight worm burden, mainly *Haemonchus contortus* and *Trichostrongylus* spp., this was almost eliminated in the early periods by the administration of phenothiazine and maintained at negligible levels thereafter. Owing to the system of pen hygiene made possible by the design and structure of the sheep house, there was very little risk of re-infestation, and the sheep may be considered to have been observed throughout under almost worm-free conditions.

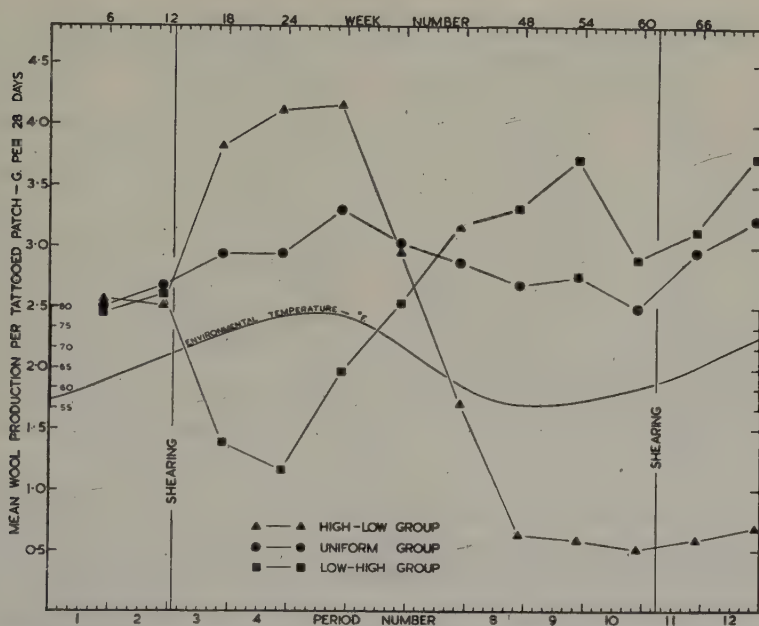


Fig. 7.—Corriedale Series. The mean wool production per tattooed patch, expressed as dry weight per unit time.

Apart from the cases mentioned above, all sheep survived the experiment without disturbance other than from minor extraneous causes. Very brief periods of inappetence were caused in two Corriedales, Nos. 250 and 257, the former by a transient abscess and the latter by a very mild fly-strike. In both cases, appetite had returned to normal within five days.

III. THE INFLUENCE OF FACTORS OTHER THAN THE PLANE OF NUTRITION ON WOOL-GROWTH RATE

(a) Experimental Results

The data for the Uniform groups revealed that influences other than the plane of nutrition were causing between-period variation in wool-growth rate. Analysis of this variation was necessary before the relation of wool-growth rate to the nutrient intake in the High-Low and Low-High groups could be adequately considered.

Partial regression analyses of the mean values for the Uniform groups show the dependence of wool-growth rate, fibre cross-sectional area, and fibre length, on atmospheric temperature, time (in periods of 6 weeks), fleece weight, and

TABLE 6

REGRESSION OF WOOL-GROWTH RATE (G. PER SHEEP PER DAY) ON ATMOSPHERIC TEMPERATURE, TIME, AND NITROGEN INTAKE FOR INDIVIDUAL SHEEP IN THE UNIFORM GROUPS†

Breed	Sheep No.	Independent Variate		
		Atmospheric Temperature (°F.)	Time (weeks)	Nitrogen Intake (g. per day)
Camden Park	225	$0.0490 \pm 0.0119^{**}$	0.0383 ± 0.0265	0.1878 ± 0.1391
	226	$0.0526 \pm 0.0089^{**}$	-0.0102 ± 0.0250	-0.2219 ± 0.1101
	228	$0.0532 \pm 0.0204^*$	0.0249 ± 0.0447	0.0234 ± 0.1491
Merino	240	$0.0518 \pm 0.0098^{***}$	0.0408 ± 0.0224	-0.0412 ± 0.0757
Corriedale	243	$0.0835 \pm 0.0197^{**}$	$0.2958 \pm 0.0422^{***}$	$0.2440 \pm 0.0688^{**}$
	245	$0.1134 \pm 0.0207^{***}$	$0.1684 \pm 0.0462^{**}$	$0.5019 \pm 0.1268^{**}$
	247	$0.0601 \pm 0.0169^{**}$	0.0816 ± 0.0372	-0.2244 ± 0.1143
	253	$0.0928 \pm 0.0186^{**}$	-0.0091 ± 0.0410	0.1820 ± 0.1276

* $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

† In the original partial regression analyses for this table, the term for fleece weight was omitted because in no case was there a significant association in the analysis of the group means (Table 7).

nitrogen intake (Table 7). The independent variate, fleece weight, represents the total fleece weight carried by the sheep calculated for each period by a proportionate division, based on sample fleece-growth rate of the fleece weight measured at shearing time. The term nitrogen intake was included to account for variation in the nitrogen percentage of the ration and occasional feed residues left by some sheep.

A further analysis was made of the regression of wool-growth rate on atmospheric temperature, nitrogen intake, and time by calculating the coefficients for the individual sheep in the Uniform groups (Table 6). The data on which these regression analyses were made are illustrated in Figures 6-11.

Table 7 shows statistically significant regression coefficients for the effect of atmospheric temperature on wool-growth rate and on fibre length for both breeds.

The effect of the environmental temperature on wool-growth rate, after adjustment for other variables would amount to a range of 44.1 per cent (Camden Park Merino) and 23.5 per cent. (Corriedale) of the mean wool-growth rate for the observed temperature range of 55° to 80°F. Wool-growth rate adjusted for the other variables is shown plotted against temperature in Figure 18.

The coefficients for fibre cross-sectional area and fibre length indicate that the effect of temperature on wool-growth rate was brought about by a change in length-growth rate rather than by a change in fibre thickness.

Table 6 shows that the regression of wool-growth rate on atmospheric temperature is positive and significant for each individual sheep.

Table 7 shows significant negative coefficients for the regression of fibre cross-sectional area on fleece weight in both breeds. Correspondingly, the coefficients for fibre lengths are positive in both breeds, significant for the Camden Park Merinos but not quite significant for the Corriedales.

TABLE 7

REGRESSION OF WOOL-GROWTH RATE ON ATMOSPHERIC TEMPERATURE, FLEECE WEIGHT, TIME, AND NITROGEN INTAKE FOR THE UNIFORM GROUPS

Breed	Independent Variate	Dependent Variate		
		Wool Weight (g. per day)	Fibre Cross-Sectional Area (μ^2)	Fibre Length (cm. per day)
Camden Park Merino	Atmospheric temperature ($^{\circ}\text{F.}$)	$0.0578 \pm 0.0110^{**}$	0.484 ± 0.422	$0.00802 \pm 0.000138^{***}$
	Fleece weight (lb.)	0.0478 ± 0.0567	$-6.724 \pm 2.169^*$	$0.00199 \pm 0.000706^*$
	Time (periods)	0.0157 ± 0.0175	$-2.337 \pm 0.670^*$	$0.000642 \pm 0.000218^*$
	Nitrogen intake (g. per day)	-0.0462 ± 0.1032	4.126 ± 3.951	0.00170 ± 0.001287
Corrie- dale	Atmospheric temperature ($^{\circ}\text{F.}$)	$0.0818 \pm 0.0123^{***}$	0.391 ± 0.587	$0.000601 \pm 0.000124^{**}$
	Fleece weight (lb.)	-0.0342 ± 0.0289	$-7.296 \pm 1.383^{**}$	0.000534 ± 0.000291
	Time (periods)	$0.1382 \pm 0.0208^{***}$	0.439 ± 0.994	$0.001072 \pm 0.000209^{**}$
	Nitrogen intake (g. per day)	$0.1908 \pm 0.0579^*$	3.147 ± 2.771	0.001201 ± 0.000583

* $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

The effect on fibre cross-sectional area amounts to decreases of $6.72\mu^2$ (Camden Park Merino) and $7.30\mu^2$ (Corriedale) per pound increase in fleece weight. Thus, the effect of fleece weight on fibre cross-sectional area after adjustment for other variables would amount to 13.5 per cent. of the mean cross-sectional area in the Camden Park Merinos and 11.8 per cent. in the Corriedales, for the ranges in weight shown by the two breeds in this experiment. The mean fibre cross-sectional area for each period, adjusted for other variables, is shown plotted against fleece weight in Figure 19. The adjustments were applied for the sake of uniformity though some variables had no significant effect.

The individual analyses (Table 6) show a variable effect of time in the different sheep. The time change in wool-growth rate is significant only for two of the Corriedale sheep. The trend is positive and it is concluded that these two sheep had not quite reached their mature wool-producing capacity.

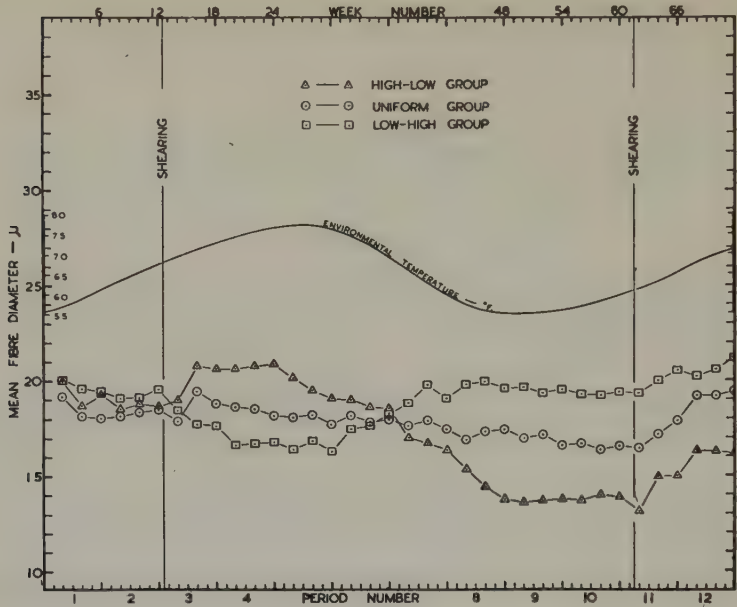


Fig. 8.—Camden Park Merino Series. The mean fibre diameter measured at a fixed position on the side immediately adjacent to the tattooed patch.

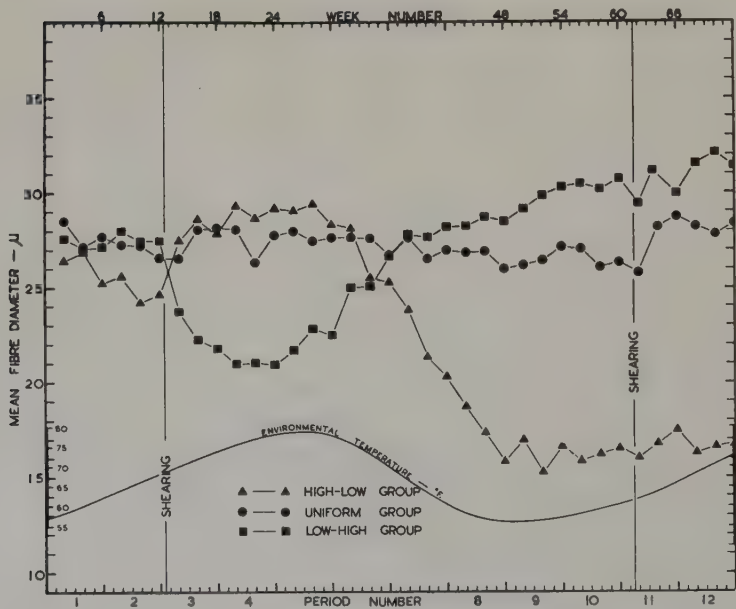


Fig. 9.—Corriedale Series. The mean fibre diameter measured at a fixed position on the side immediately adjacent to the tattooed patch.

(b) *Discussion*

There appears to be no reference in the literature to an unequivocal positive association between environmental temperature and hair growth in the mammalian coat. Strangeways (1933) observed an immediate decrease in the amount of hair produced when guinea pigs were removed from an environment of 60-70°F. and placed in a room with a temperature of 40-50°F. The production of hair remained at a minimum for 2 or 3 weeks and then returned to its normal level. Eaton and Eaton (1937) found a positive correlation between length-growth rate of hair on the human face and atmospheric temperature but did not observe whether the weight of hair produced was associated with the temperature. According to Marston (1935) external temperature changes "providing these were not great," did not have any measurable effect on wool-growth rate on frequently shaven skin patches such as we have employed in this work. Marston does not, however, say within what limits of temperature change his statement is intended to apply.

Our observation of a positive association between wool-growth rate and atmospheric temperature may appear inconsistent with the familiar appearance of the winter coat in furred animals. In the latter instance, however, one must remember that the hair fibres are not continually growing but attain their full length, enter a quiescent period, and are then shed (Trotter 1924; Dawson 1930; Strangeways 1933). The growth of the winter coat appears to be under the control of a neuro-endocrine mechanism (Bassett, Pearson, and Wilke 1944; Bissonnette and Bailey 1944) and this may not affect the production of continuously growing hair or wool. We suggest that our observed association between wool-growth rate and atmospheric temperature may be due to changes in the blood circulation of the skin in the course of its normal responses as part of the heat regulating mechanism of the body.

Although it has not been specifically demonstrated in the sheep, cutaneous vasodilatation with accompanying increase in blood volume and cardiac output has been demonstrated in various animals as a means of increasing heat loss by radiation and insensible water loss in hot environments (Barcroft *et al.* 1923; Pickering 1932; Freeman and Zeller 1937; Gagge, Winslow, and Herrington 1938; Grant and Holling 1938; Sunderman, Scott, and Bazett 1938; Hick, Keeton, and Glickman 1939; Thauer 1939; Scott, Bazett, and Mackie 1940; Barcroft and Edholm 1943; Naide 1944; Sams 1944; Spealman 1944; Ralston and Ken 1945; Spealman 1945; Barcroft, Bonnar, and Edholm 1947). Although there is apparently some dilution, the phenomenon appears to be largely one of redistribution of the circulating blood. Values of blood non-protein nitrogen which, with sheep on the same ration, may be taken as a crude measure of the blood concentration of wool substrate, showed no association with atmospheric temperature in the Uniform groups.

Increased hair growth on a circumscribed area associated with lesions of the nerves supplying it has been frequently reported. The phenomenon has been explained on the assumption that owing to functional interruption of the sympathetic innervation of the vessels in question (Kuntz 1945) the papillary blood supply has been increased. Kronacher and Lodemann (1930) quote a case in which resection of the sympathetic trunk on one side in the neck of a monkey led to an increased growth of the head hairs on the same side.

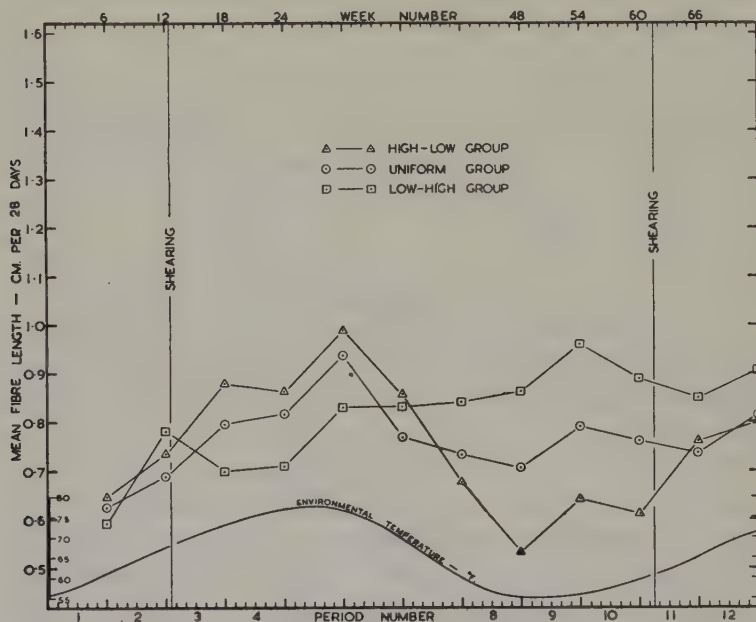


Fig. 10.—Camden Park Merino Series. The mean fibre length expressed as centimetres per unit time computed from wool weight and fibre diameter.

Haddow and Rudall (1945) demonstrated that the waves of active hair growth in the rat are associated with corresponding waves of vasodilatation.

The empirical evidence of an association between atmospheric temperature and wool-growth rate thus confirms the ample physiological expectation of such an association. However, the possibility of this phenomenon occurring has seldom been taken into account sufficiently in quantitative studies of wool growth.

The detailed mechanism of the circulatory responses of the skin to changes in the thermal environment will not be discussed in this paper. However, it should be noted that the relatively greater exposure of the periodically clipped fleece sample area to the thermal environment possibly causes a greater circulatory change on the sample area than over the remainder of the wool-bearing surface. This may be expected from the operation of local spinal and axon reflexes. The use of a constant ratio for converting growth rate of fleece sample to total fleece-

growth rate implies, therefore, a proportionality between sample and total fleece-growth rate which may not hold for all atmospheric temperatures. The ratios have been used nevertheless to render the data in the present analysis more comparable with the data used to relate total fleece-growth rate to the plane of nutrition.

It cannot be overlooked that an additional mechanism may be concerned in the relation of wool-growth rate to atmospheric temperature. Cutaneous vasodilatation besides providing an increased nutrient supply to the follicle also causes an increase in skin-surface temperature which may increase the activity of enzymes concerned in the synthesis of wool keratin.

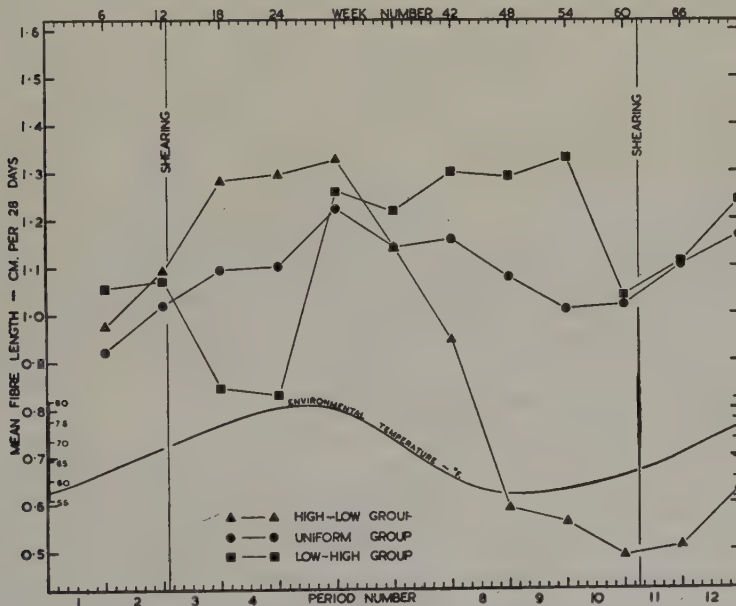


Fig. 11.—Corriedale Series. The mean fibre length expressed as centimetres per unit time computed from wool weight and fibre diameter.

The expression of the temperature effect almost wholly in increased length-growth rate may be explained by an increase in tissue hydrostatic pressure resulting from cutaneous vasodilatation. The increased follicle growth activity in taking the path of least resistance may be prevented from appearing as an increase in fibre thickness. Increased sweat-gland activity associated with increased atmospheric temperature may also contribute to an increase in tissue pressure. Furthermore we may note that changes in atmospheric temperature over the range observed did not cause any appreciable change in body weight (Figs. 4 and 5), when compared with the effect of the plane of nutrition. Consequently, an increase in atmospheric temperature is unlikely to cause an increase in surface area which would allow the fibre to expand laterally with increased follicle activity.

Similarly, the inverse association between fibre thickness and fleece weight may be explained as resulting from the effect of increasing fleece covering on the moisture content of the skin causing increased tissue hydrostatic pressure and deformation of the follicle. Rudall (1935) observed that removal of part of the fleece caused increased medullation of fibres on the shorn areas. However, he did not at the same time measure fibre thickness, and it is impossible to say what association between this dimension and atmospheric temperature was present in his sheep.

IV. THE RELATION OF WOOL-GROWTH RATE TO THE NITROGEN INTAKE

(a) Experimental Results

The influence of the plane of nutrition on wool-growth rate is summarized in Figures 6 to 13 in which the mean value for each group of wool weight, fibre diameter, fibre length, and number of fibres per sample area is plotted on a time scale. The mean atmospheric temperature for each period is also shown on each graph and the association of wool-growth rate with atmospheric temperature revealed by the earlier regression analysis is illustrated by the graphs. The influence of fleece weight on fibre thickness and fibre length is also shown, although the interpretation of the data for the latter three periods is complicated by the occurrence of inappetance associated with the feeding of the new mixture in Period 10 (Figs. 2 and 3).

The unexpectedly close association between atmospheric temperature and wool-growth rate seriously hampers the analysis of the relation of wool-growth rate to the nitrogen intake. Owing to the confounding effect of atmospheric temperature, the data for the High-Low and Low-High groups cannot be used to supply estimates for the individual sheep of the constants in equations (1) and (2). The group mean data for Periods 4 and 9 are free from this objection but only supply values of wool-growth rate at two planes of nutrition above maintenance, whereas three sets of values are necessary to estimate k , k' , A , and x_0 . However, inspection of the data revealed that the value of x_0 was probably very small, in which case, without serious bias, values of k , k' , and A could be estimated from two sets of values by assuming that x_0 was equal to zero.

Owing to the differences between the Camden Park Merino groups in productive capacity, as defined by the values for wool-growth rate obtained in Period 2, the data for these groups were adjusted by the regression of Period 4 and 9 values upon those of Period 2. The data for Periods 4 and 9 for each breed were then combined, giving values of wool-growth rate corresponding to the mean atmospheric temperature of the two periods, 64.6° F. (Camden Park Merino) and

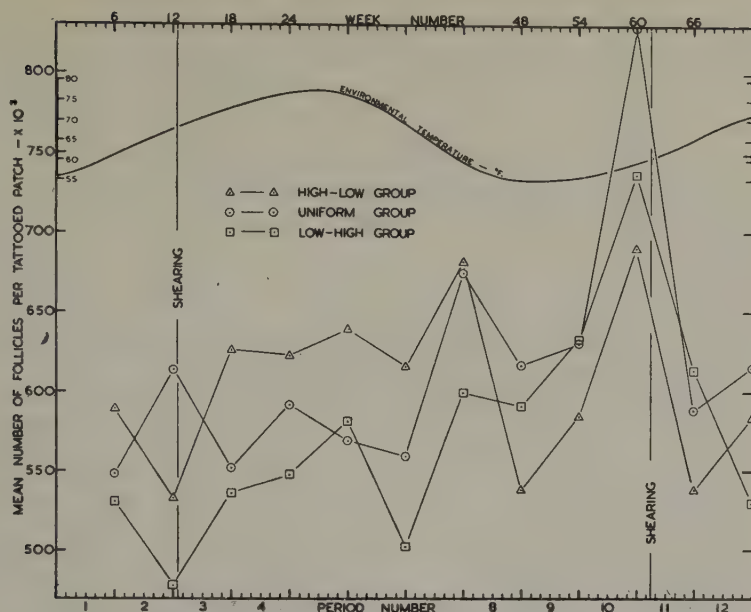


Fig. 12.—Camden Park Merino Series. The mean number of follicles per tattooed patch in thousands computed from periodic density estimates and measurements of the skin patch relaxed with the sheep standing.

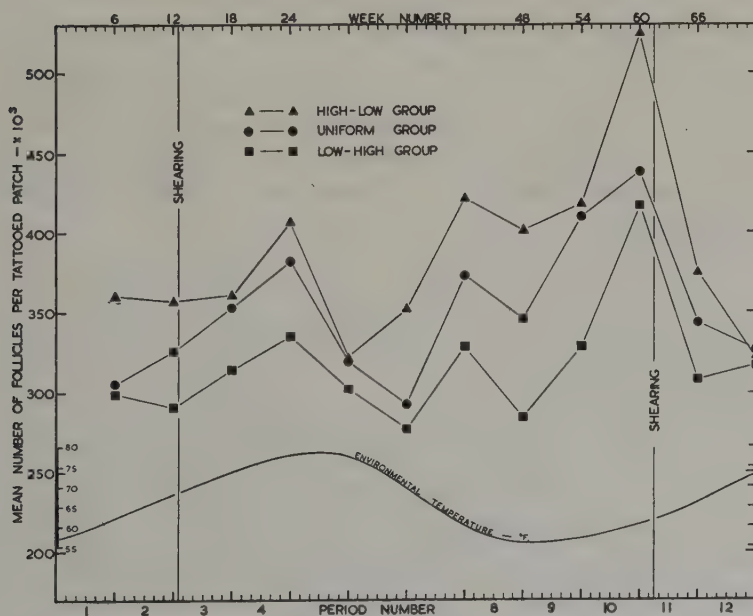


Fig. 13.—Corriedale Series. The mean number of follicles per tattooed patch in thousands computed from periodic density estimates and measurements of the skin patch relaxed with the sheep standing.

66.1° F. (Corriedale). Owing to the variable effect of atmospheric temperature on wool-growth rate in individual sheep (Table 6) it was not considered desirable to fit the equation separately to the Period 4 and the Period 9 data to obtain the effect of the temperature difference between these periods on the value of the constants. Also, further information on the effect of atmospheric temperature on the ratio of total to sample wool-growth rate is required before the effect of atmospheric temperature on the constants of equation (2) can be properly determined.

TABLE 8
THE REGRESSION OF CHANGE IN WOOL-GROWTH RATE PER UNIT CHANGE IN ATMOSPHERIC TEMPERATURE UPON THE NITROGEN INTAKE

Sheep No.	Camden Park Merino		Sheep No.	Corriedale	
	Regression Coefficient	Standard Error		Regression Coefficient	Standard Error
227	0.003985 ± 0.000413***		246	0.006476 ± 0.001736*	
232	-0.000379 ± 0.002238		250	0.006987 ± 0.002192*	
234	0.000854 ± 0.001802		251	—	
241	0.007031 ± 0.001748*		257	0.002832 ± 0.001658	

* $P < 0.05$. *** $P < 0.001$.

It is convenient to fit equation (1) to the data and to determine the value of A and k . k' is then given by the product of k and A . Equation (1) may be fitted by selecting a value of A and calculating the corresponding values of k for each of the two points. By successive approximation, the value of A corresponding to equal values of k for the two points is readily found.

The values found for the parameters A , k , and k' are shown in Table 9. The difference between the breeds in the value of k' cannot be regarded as significant. The data do not therefore dispute the hypothesis embodied in equation (2) generalizing the relation between wool-growth rate, nitrogen intake, and wool-producing capacity.

TABLE 9
ESTIMATES FROM THE EXPERIMENTAL DATA OF THE PARAMETERS IN EQUATIONS (1) AND (2)

Breed	A	k	k'
Camden Park Merino	5.3	0.092	0.487
Corriedale	21.9	0.019	0.422

As pointed out above, the fit of equations (1) and (2) to the data for the individual sheep in the High-Low and Low-High groups is complicated by the parallel change in nitrogen intake and atmospheric temperature. The effect of the two factors is synergistic in the High-Low group and antagonistic in the Low-High group. Furthermore, the wool-growth rate of the Low-High groups did not, in the intermediate periods, appear to be in equilibrium with the nitrogen intake. However, assuming the estimates of k' from the group means to

be valid for the individuals, the individual data may be used to supply estimates of the influence of the plane of nutrition on the effect of atmospheric temperature on wool-growth rate on the sample area. The individual data of the High-Low groups were used for this purpose.

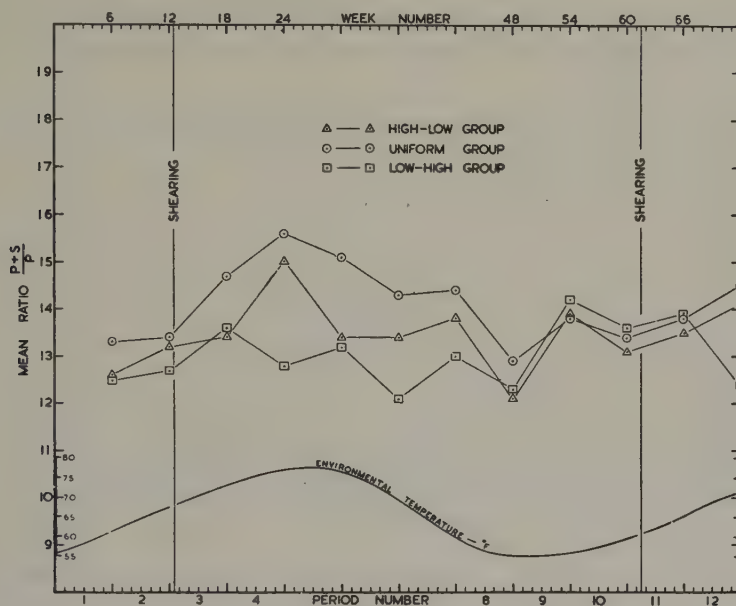


Fig. 14.—Camden Park Merino Series. The mean size of the hair follicle group measured by the ratio of the total follicle population (primary and secondary) to the primary follicle population.

The mean value of k' for both breeds, 0.454, estimated from the data of Periods 4 and 9, was used to estimate values of A for each sheep in the High-Low groups from the data of Period 2. The atmospheric temperature in Period 2 closely corresponds to the mean value for Periods 4 and 9. The theoretical wool-growth rate corresponding to the nitrogen intake in each period of each of the sheep in the High-Low group, was then calculated. The deviation of the actual wool-growth rate from the theoretical values may be ascribed to the temperature differences between each period and the mean value for Periods 4 and 9. The deviations in wool-growth rate were divided by the corresponding temperature deviations to provide estimates of the regression coefficient of wool-growth rate on atmospheric temperature for the planes of nutrition of the particular periods.*

The relation of this change per degree of temperature ($^{\circ}\text{F}.$) to the nitrogen intake for the individual sheep in the High-Low groups is shown in Figures 20 and 21. There is some indication that the influence of environmental temperature

* Only the data for Periods 2 to 9 were used for reasons given earlier. The relation for sheep No. 251 was omitted. This animal was sick in Period 6 and died in Period 7. The data for Periods 6 and 7 for sheep No. 257 were also omitted because of sickness in these periods.

on wool-growth rate increases with the plane of nutrition (Table 8). However, in these sheep, the higher levels of feeding occurred at the higher temperatures and *vice versa* so that from the data of the High-Low groups we cannot associate the apparent effect unequivocally with the plane of nutrition alone. For the present analysis, however, which aims at an adjustment for temperature to permit the fitting of curves for response to planes of nutrition, the present assumption will serve the purpose. A more detailed consideration will be given to the point in a later paper.

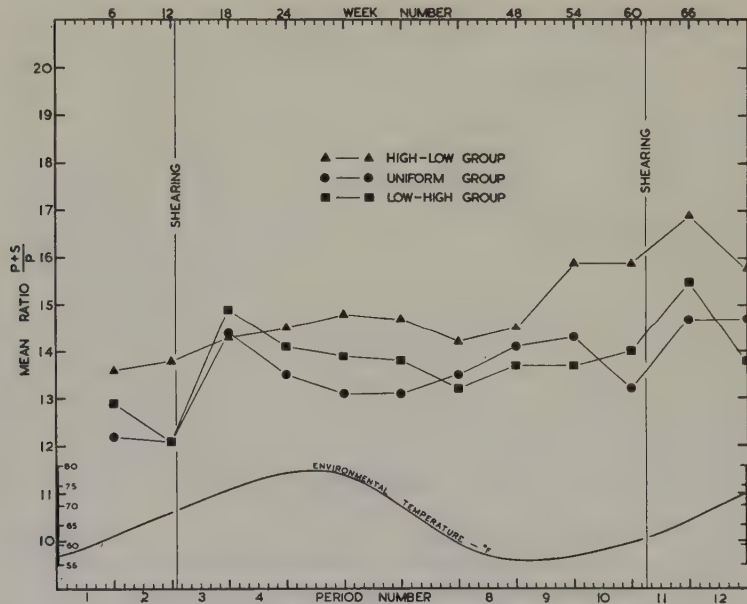


Fig. 15.—Corriedale Series. The mean size of the hair follicle group measured by the ratio of the total follicle population (primary and secondary) to the primary follicle population.

Straight lines were considered an adequate fit to the data of Figures 20 and 21. Adjustments to wool-growth rate for variation in atmospheric temperature were made by reading from the fitted line in each case the appropriate value per °F. change in temperature corresponding to each level of nitrogen intake. The fit of equation (2) to the individual data of the High-Low groups after this adjustment is shown in Figures 22 and 23.

If now the blood values for non-protein nitrogen be taken as a crude measure of the concentration of circulating wool substrate, it appears from the analyses that the influence of the plane of nutrition on wool-growth rate is due largely to a change in the blood concentration of these materials. Here is a contrast to the mechanism postulated for the influence of atmospheric temperature on wool growth, namely, changes in the rate and volume of the cutaneous blood flow. Figures 16 and 17 show the mean blood protein nitrogen of the six groups throughout the experiment.

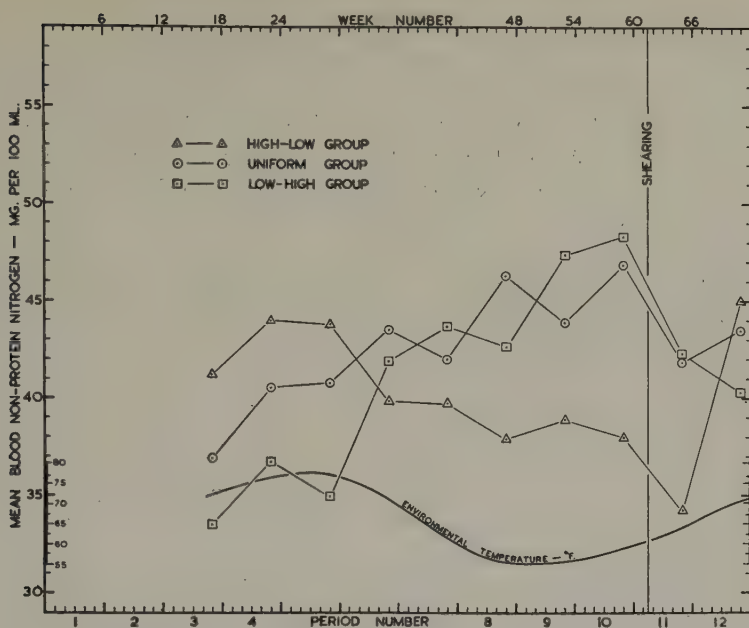


Fig. 16.—Camden Park Merino Series. The mean values for blood non-protein nitrogen.

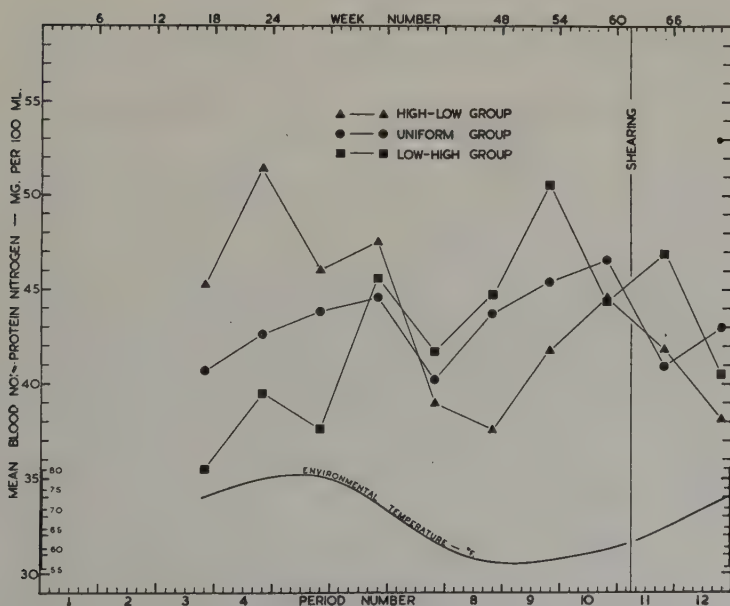


Fig. 17.—Corriedale Series. The mean values for blood non-protein nitrogen.

It may be noted in Figures 6 to 15 that the influence of the plane of nutrition on wool-growth rate is expressed mainly through changes in fibre-thickness and fibre-length growth rate with little evidence of change in the number of active follicles. From Period 8 onwards, however, there is slight evidence in Figure 12 of a decrease in the number of active follicles in the Camden Park Merino High-Low group, and by Period 12 in the corresponding Corriedale group also. This suggestion is supported by the histological picture in both of these groups, although the number of inactive follicles is small. In Figures 14 and 15, the ratio of total to primary follicles for each group throughout the experiment is shown. The graphs do not indicate any consistent effect of the plane of nutrition.

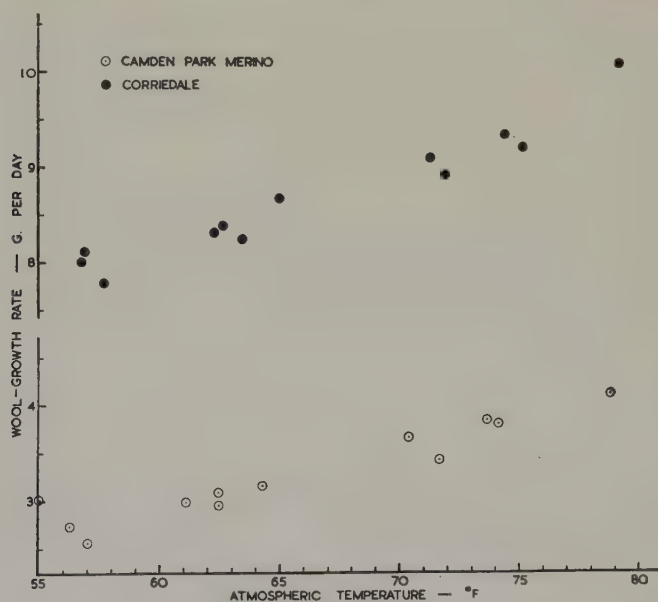


Fig. 18.—The relation of wool-growth rate (adjusted for the other variables) to atmospheric temperature.

From consideration of the factors affecting the relative expression of changes in wool-growth rate as changes in fibre thickness and length, it is apparent that the inherent fibre thickness and length of a fleece are not as precisely defined by the nitrogen intake and atmospheric temperature as is the total wool-growth rate. The generalization expressed in equation (2) can not be applied to the relations of fibre thickness and fibre-length growth to the nitrogen intake. The whole question of the responses in individual skin and fleece characters will be treated in another paper.

(b) Discussion

Although the close approximation of the values of k' for two contrasting sheep types is promising evidence that the same value may apply to other types, this conclusion is insecure without further experimental work. In any case, the

generality of a particular value of k' is limited to relationships in which the nitrogen is supplied from a particular diet. Indeed, the value of k' may be taken as a broad measure of the availability for wool growth of the dietary nitrogen. It is not to be expected, therefore, that data could be found in the literature which would be strictly comparable with the relationship expressed by equation (2).

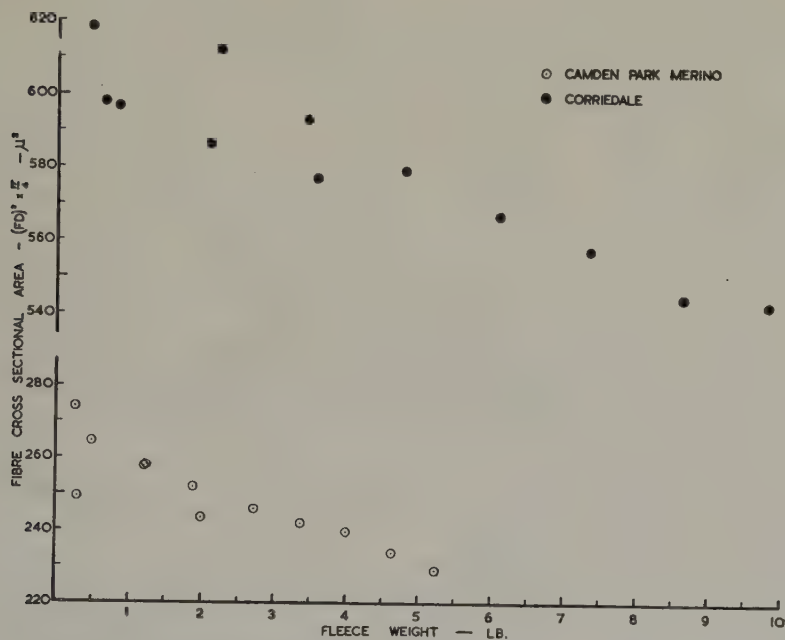


Fig. 19.—The relation of fibre cross-sectional area (adjusted for the other variables) to fleece weight.

Furthermore, in view of the apparent effect of environmental temperature on wool-growth rate, data obtained from sheep kept at different planes of nutrition at different seasons of the year need appropriate adjustment. In our experience, a period of about three months may be required before equilibrium between wool-growth rate and nitrogen intake is established after the plane of nutrition has been radically altered. Consequently, unless the animals be housed in constant temperature rooms, large seasonal differences in mean air temperature must be expected in experiments of this kind. The effects of this temperature fluctuation on wool-growth rate are important where the same sheep are being studied at different planes of nutrition. It is of lesser importance when several individuals or groups of sheep are being compared simultaneously at different planes of nutrition. It is of considerable importance if data from experiments at different laboratories are being studied.

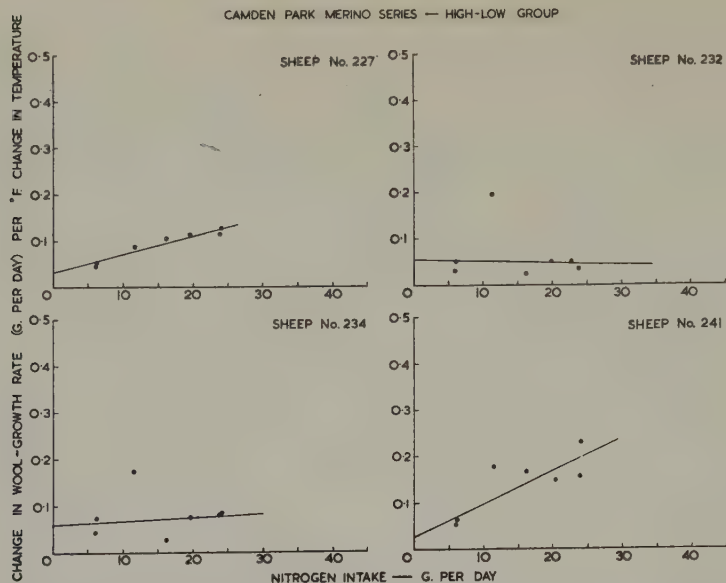


Fig. 20.—Camden Park Merino Series. The relation of the rate of change of wool-growth rate per degree of atmospheric temperature to nitrogen intake for the individual sheep in the High-Low group.

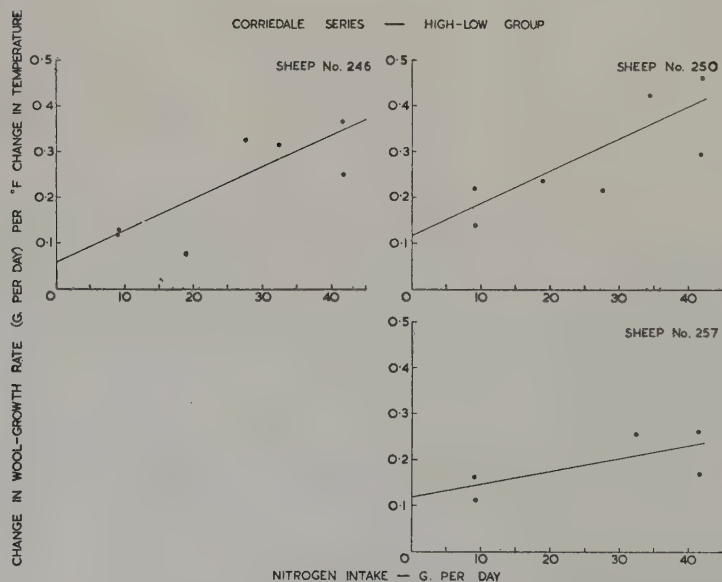


Fig. 21.—Corriedale Series. The relation of the rate of change of wool-growth rate per degree of atmospheric temperature to nitrogen intake for the individual sheep in the High-Low group.

However, data from two examples in the literature may be compared with the relationship given in equation (2) using the mean value of k' for both breeds, 0.454, and explaining deviations in terms of the factors discussed above.

TABLE 10
COMPARISON OF DATA DERIVED FROM LITERATURE WITH THEORETICAL VALUES
CALCULATED FROM EQUATION (2)

Reference	Sheep No.	A* Value (g. per day)	Nitrogen Intake (g. per day)	Actual Wool Growth (g. per day)	Theoretical Wool Growth (g. per day)
Maré and Bosman (1934): South African Merino Wethers. Age: 3 yr.	---	6.8	9.7 12.7 13.9 16.9	3.5 4.0 3.9 4.6	3.2 3.9 4.1 4.6
Marston (1948): South Australian Merino Ewes. Age: 3½ yr. (Ascending Series)	522	17.1	3.67 8.08 12.90 19.83	1.5 1.4 4.6 7.0	1.6 3.3 5.0 7.0
	547	21.5	3.48 7.86 12.51 19.26	1.3 1.7 3.5 7.2	1.5 3.3 5.0 7.2
	558	49.0	3.38 7.97 12.60 18.20	1.1 1.4 4.8 7.6	1.5 3.5 5.3 7.6
Marston (1948): South Australian Merino Ewes. Age: 3½ yr. (Descending Series)	550	6.6	4.18 9.55 15.05 18.85	1.0 2.0 3.2 4.8	1.7 3.2 4.3 4.8
	559	12.4	3.95 9.42 15.55 20.30	1.6 1.7 4.4 6.5	1.7 3.6 5.4 6.5
	572	136.0	4.62 9.42 15.20 19.80	1.5 1.7 7.1 8.7	2.1 4.2 6.7 8.7

* A values were calculated from nitrogen intake and wool-growth values at the highest plane of nutrition for each series of data.

Maré and Bosman (1934) kept two groups each of 11 Merino wethers for 9 months, one on a high and one on a low plane of nutrition. For the next 9 months each original group was divided. For one subgroup the original treatment was maintained and for the other it was reversed. Mean values of wool-

growth rate observed for these four series of sheep during the 18 months of this experiment are set out in Table 10 with the theoretical values computed from equation (2). Although the data in this experiment were obtained with

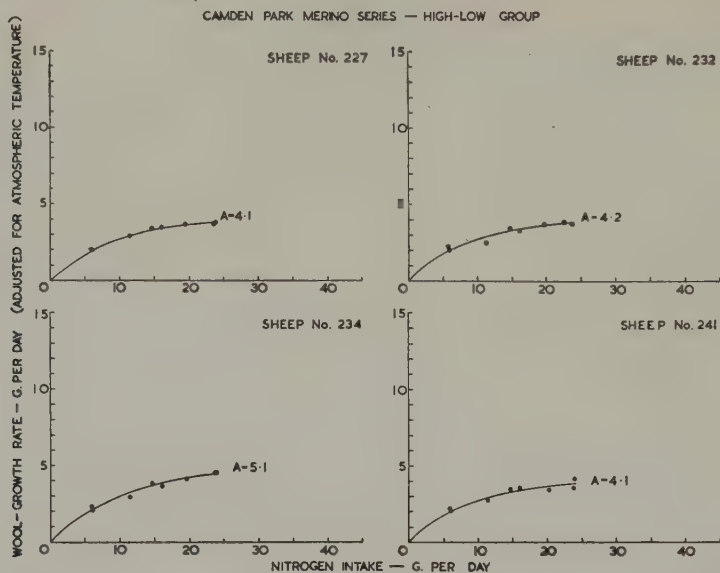


Fig. 22.—Camden Park Merino Series. The fit of equation (2) to the data (adjusted for atmospheric temperature) from individual sheep in the High-Low group.

a diet of different qualitative composition at high and low planes of nutrition and under unknown conditions of environmental temperature, there is reasonable agreement between observed and expected values. Environmental temperature fluctuations are, in this case, comparable for all 4 groups although unknown in relation to the conditions of our experiment.

Marston (1948*a*, 1948*b*) gives values of wool-growth rate and nitrogen intake at 4 planes of nutrition for 6 South Australian Merino ewes. These data were obtained by taking 3 of the sheep from a high to a low plane and, simultaneously, 3 sheep from a low to a high plane of nutrition. The sheep were kept for 10 weeks on each plane of nutrition and wool-growth rate with complete energy and nitrogen balance sheets determined during the latter 14 days of each such period. The observed values and these to be expected from the application of equation (2) are also set out in Table 10. In this case the two series of values do not agree very closely. Discrepancies may perhaps be explained partly by the seasonal trends in temperature of unstated magnitude occurring simultaneously with the changing planes of nutrition; partly, by the negative nitrogen balance of Marston's sheep at the lowest plane of nutrition; partly, by differences in the qualitative composition of the diet, perhaps requiring a different value of

k' ; and partly by slight differences in the method of estimating the wool-growth rate for each sheep from that used by us.

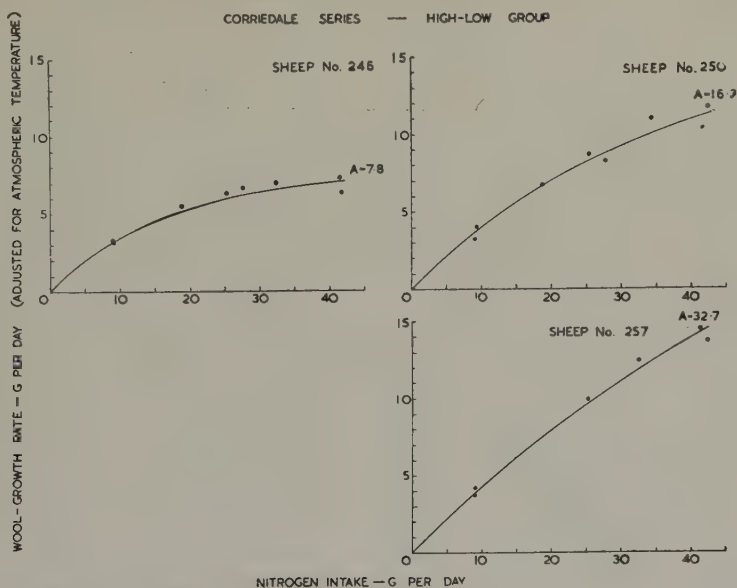


Fig. 23.—Corriedale Series. The fit of equation, (2) to the data (adjusted for atmospheric temperature) from individual sheep in the High-Low group.

Figure 24 shows the theoretical curves derived from equation (2) relating wool-growth rate to nitrogen intake for sheep of different wool-producing capacities. The curves fan out from the origin, differences in wool-growth rate between sheep of different producing capacity becoming progressively greater as the nitrogen intake increases. It has been assumed here, as it has been in deriving k , k' , and A from our data, that $x_0 = 0$ in which case equation (2) may be written

$$y/A = 1 - e^{-k'(x/A)}.$$

This assumption seems reasonable on inspection of the data but rigorous proof must await further experiments. It is conceivable that the value of x_0 , although never large, is different for different individuals and is also influenced by the thermal environment. If this be true, it demonstrates that the assumption of $x_0 = 0$ made in this paper is an oversimplification. However, the comparison of the estimates of the parameters k , k' , and A made for the two breeds is relatively unaffected by inaccuracy in the estimate of x_0 . It is clear from Figure 24 that estimates of the asymptote A from single values of wool-growth rate and nitrogen intake using any predetermined value of k' become increasingly more accurate as the plane of nutrition rises. Further, there is no reason to suppose that the

standard error of measurement of wool-growth rate increases with the level of feeding sufficiently to nullify this consideration. In fact, it is more probable that the error decreases with an increasing quantity of wool measured.

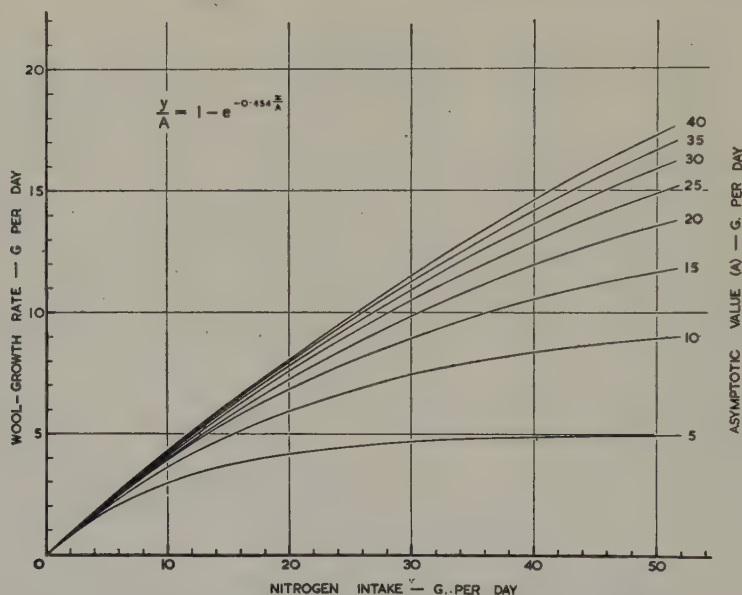


Fig. 24.—Theoretical curves derived from equation (2) relating wool-growth rate to nitrogen intake for sheep of different wool-producing capacities.

Figure 24 also demonstrates that the value of A is not a linear function of the corresponding values of wool-growth rate at ordinary levels of feeding. The value of A increases at a much greater rate than the corresponding wool-growth rate at a given nitrogen intake. For example, a difference in A value of 5 between 10 and 15 corresponds to a difference in wool-growth rate of 2.1 g. per day at a nitrogen intake of 40 g. per day. However, at the same nitrogen intake level, a difference in A value of 5 between 35 and 40 corresponds to a difference in wool-growth rate of only 0.4 g. per day. Thus the high values of A calculated for sheep Nos. 558 and 572 in Table 10 do not reflect proportionately large wool-growth rates at ordinary levels of feeding. Nor, in this case, can the values of A be taken as accurate estimates of productive capacity owing to the doubtful validity of applying equation (2) to Marston's data.

We may consider now the measurement of inherent differences in wool-growth rate in the light of equation (2). For laboratory studies the concept of an asymptotic value of wool-growth rate characteristic of the individual provides a possible method of expressing the inherent wool-producing capacity of a sheep independent of the plane of nutrition. From our data, it would seem desirable

to feed the sheep under observation at as a high plane of nutrition as possible to obtain the most accurate estimate of A . Moreover, since inherent differences in *ad libitum* food capacity constitute an important source of variation in productive performance, it is therefore preferable to feed *ad libitum* rather than at any fixed level. The self-chosen level of food intake thus becomes an additional important observation in itself directly relevant to the study of inherited performance in the sheep. However, before these concepts can find proper application in Mendelian studies of wool growth in the laboratory, more work is required to examine further the generality of equation (2) applied to various sheep types, as well as the effect of dietary composition and environmental temperature on the values of the constants x_0 , k' , and A .

In field experimentation and in ordinary sheep breeding practice, the plane of nutrition cannot be known exactly for the individual, and the calculation of asymptotic values of wool-growth rate is virtually impossible. The observed growth rate of wool in the field under ideal pasture conditions is mainly the resultant of the separate inherent capacities for food consumption and for wool growth and the sheep's relations with its thermal environment. Wide variation in the *ad libitum* food capacity of sheep may be observed with individual feeding in the laboratory, and it is certain that equally wide if not wider variation may occur with natural grazing. The *ad libitum* food consumption of the Camden Park Merinos prior to beginning this experiment varied from 680 to 1250 g. of dry matter per day for the twelve individuals. The values for the twelve Corriedales varied from 1290 to 1950 g. per day. Wider variations may be expected in larger numbers of sheep. Assuming the nitrogen content of the dry matter in these cases to be reasonably high, say 3.0 per cent., these intakes provide 20.4 to 37.5 g. of nitrogen per day for the Camden Park Merinos and 38.7 to 58.5 g. per day for the Corriedales. From Figure 24 it is clear that such variation of intake in the Camden Park Merinos ($A = 5.3$ g. per day) would cause little variation in wool-growth rate, whereas in the Corriedales ($A = 21.9$ g. per day) the variation would be considerable. It is apparent, therefore, that studies under natural grazing conditions of the mode of inheritance of wool-producing capacity in sheep are likely to prove most unsatisfactory.

Selection of livestock under the conditions in which they are most commonly maintained, is a well-accepted aim in animal breeding. When sheep are kept at a moderately high plane of nutrition for any considerable part of the average year, such as in the most favourable Merino areas of Australia, desirable characters sought by the breeder include a high inherent wool-producing capacity (A value) and a high capacity and desire for food. Such characters would be poorly expressed when sheep are maintained at a low plane of nutrition with restricted grazing opportunity such as will occur in all areas during adverse seasons. With such unfavourable conditions, the selection of sheep for expected high production performance when the seasons improve, is likely to be relatively ineffective.

V. GENERAL CONCLUSIONS

The genetic component of wool-producing capacity in the sheep is difficult to assess. Differences in the biotic environment affecting the plane of nutrition by altering the amount and quality of food available, differences in the physical, especially the thermal, environment, and differences in the food capacities of individual sheep, all tend to complicate studies of the mode of inheritance of wool-growth rate and productive capacity in the sheep. The generalized equation postulated in this study as a means of relating wool-growth rate, nutrient intake, and wool-producing capacity, although requiring further research, seems to offer a more rational means of expressing the inherent capacity of a sheep for wool production than is commonly used. Considerable difficulties prevent the application of its concepts in genetic studies except under laboratory conditions. Nevertheless, in the breeding of sheep under natural grazing conditions, due cognizance must be taken of the relationships involved.

The differential effects on fibre thickness and on fibre-length growth rate of such factors as the plane of nutrition, thermal environment and amount of fleece covering, suggest that, in field experimentation, variation in either one of these two fleece characters alone cannot necessarily be regarded as reflecting a corresponding variation in total wool-growth rate. The explanation, involving differences in tissue hydrostatic pressure, postulated for the above effects, requires experimental substantiation.

The physiological mechanisms leading to changes in the moisture content of the skin which have been suggested to explain both the apparent influence of environmental temperature on total wool-growth rate and the progressive change in fibre configuration during fleece growth, offer for study a relatively unexplored field in the physiology of wool production. This implies that studies on the physiology of heat regulation in the sheep, and especially the function of the skin, must be integrated with nutritional research if a better understanding of fleece-growth phenomena is to be attained for application in genetic research.

VI. ACKNOWLEDGMENTS

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Finally, we are especially indebted to the Trustees of the Camden Park Estate and to the owner of the Corriedale Stud for their generosity in donating the experimental sheep employed in this study.

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YIELD TRENDS IN THE WHEAT BELT OF SOUTH AUSTRALIA DURING 1896-1941

By E. A. CORNISH*

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Summary

The history of the wheat industry in South Australia is reviewed to provide a background for discussion of the forms of trend observed in yield. The period chosen for examination was 1896-1941, and the analysis extends to practically the entire wheat belt, the basic territorial unit used for assessing yield being the hundred, the mean area of which in South Australia is approximately 118 square miles. As a preliminary to the evaluation of the trends it was necessary to estimate and to eliminate the effects of variations in seasonal rainfall; the statistical technique used was that of partial regression, and reasons are given for the choice of rainfall variates.

The major soil groups under cultivation are described and mapped.

The elimination of phosphorus as a limiting factor in yield coincided with the beginning of the period under review, so that in classifying the forms of trend observed it was convenient to divide the hundreds into two groups, according to whether they were opened for cultivation before or after the advent of superphosphate.

The nitrogen status of the major wheat soils is discussed, and after consideration of relevant literature, it is concluded that the nitrogen required by the crop has been drawn almost entirely from soil reserves under the exploitative systems of cropping generally employed. The wheat belt is broadly divisible into three parts:

1. Sandy, stony, and mixed mallee soils and related types in which nitrogen becomes limiting after 20-40 years of cropping, and yields subsequently decline owing to exhaustion of the reserves.
 2. Loamy mallee soils and red brown earths, where yields increase over the period 1896-1941, but at diminishing rates as nitrogen becomes limiting.
 3. Sandy and loamy mallee and transitional mallee-solonetz soils, where yield increases linearly throughout, mainly because exploitative cropping has not been in progress long enough to make its influence apparent.
- These regions constitute only a small proportion of the total area.

The economic restoration and maintenance of the nitrogen status of the wheat soils are discussed briefly.

I. HISTORICAL INTRODUCTION

Wheat-farming is the major industry of South Australia. One feature of its expansion to this predominant position is illustrated in Figure 1, which shows the annual acreage of the crop from 1836 to 1941; four clearly defined phases

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are apparent, and for the purpose of tracing the history of the industry it is

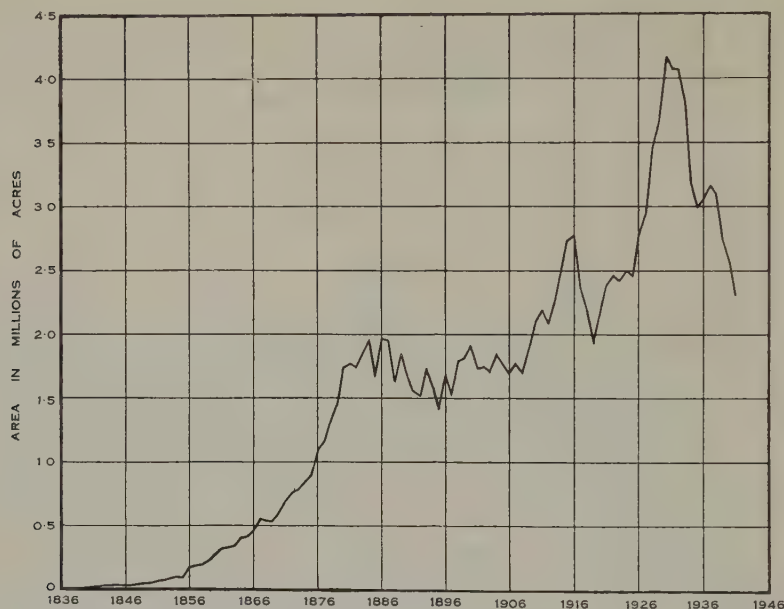


Fig. 1.—Annual acreage of wheat cultivated for grain in South Australia (1836-1941).

convenient to use them with the following end points: 1836-85, 1886-1908, 1909-32, 1933-41. This partitioning is made primarily on the basis of acreage and its temporal rate of change, but at the same time carries other factors of importance in the development. Reference to the effects of these factors is made in the notes which follow.

(a) 1836-85

The production of wheat was first attempted on the mainland in 1838, and although only European varieties were available, fair success was achieved because of the relatively favourable climate in the immediate vicinity of Adelaide. Experience soon demonstrated the correct time to sow the crop, and the area rapidly expanded to the point where production exceeded local demand.

During the decade 1841-50, three advances gave a great impetus to production:

1. The invention of the stripper in 1843; this machinery considerably reduced the labour costs of harvesting, and made possible expansion into greater areas.
2. The construction of a flour mill in 1843 stimulated production of wheat for export of flour to interstate markets.
3. The abolition of the British corn duties in 1847 encouraged production to meet an export trade with Britain.

In the early years, cropping was confined to the Mt. Lofty Ranges and their slopes to the east and west, both on account of their geographical situation and

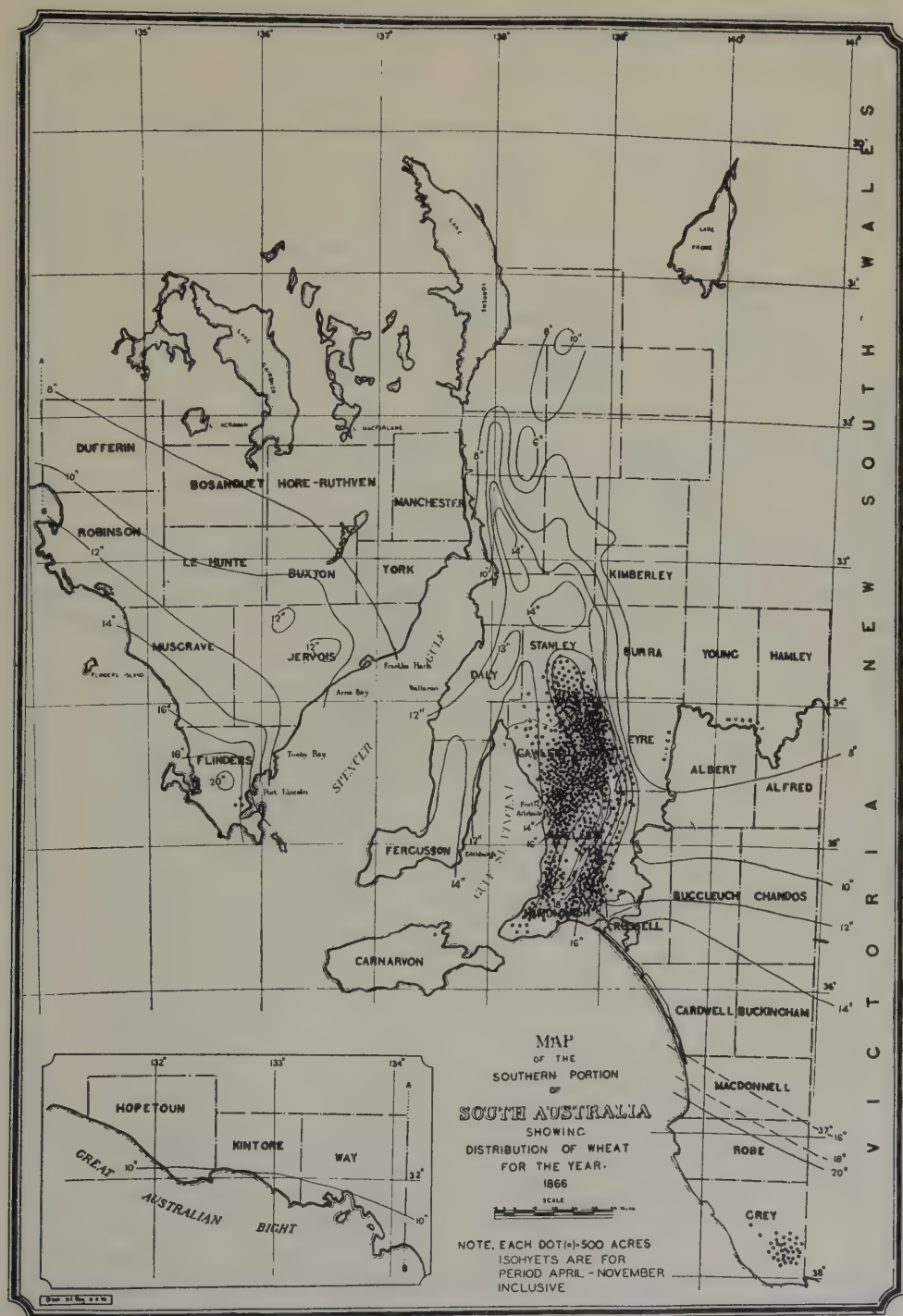


Fig. 2

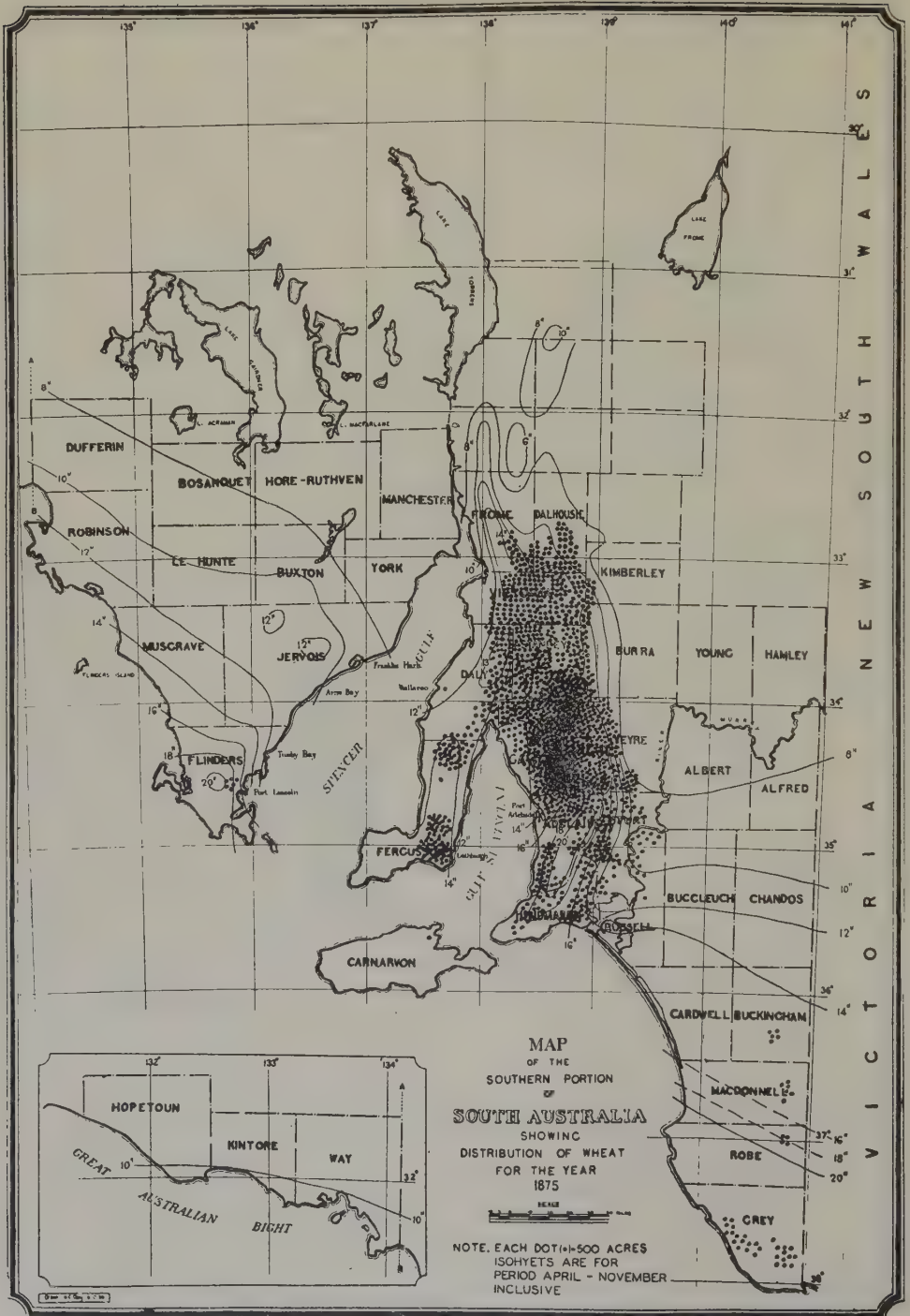


Fig. 3

the readiness with which the arable parts could be brought into production. The combined effect of these factors is illustrated in Figure 2, which shows the distribution of the area under wheat in 1866, in relation to the isohyets of seasonal rainfall (total from April to November inclusive). Over practically all land thrown open for cultivation, the rainy season was followed by a long arid period extending throughout the summer, which precluded the use of rotation systems involving wheat that had been practised successfully in Europe. As a result, a new scheme was gradually developed, in which the land lay in fallow for periods of 9-12 months prior to sowing.

As the railway northward from Adelaide was extended, and subsidiary lines were constructed, expansion of the area followed, but cropping was still restricted mainly to regions with high rainfall. The distribution in 1875 is illustrated in Figure 3. It will be observed that this diagram shows the first signs of extension into areas with less than an average seasonal rainfall of 10 in.

Increasing demands of settlement next led to an attempt to convert large areas of mallee land to wheat-farming. The high costs and difficulties of clearing that were entailed would have retarded progress but for the invention, in 1876, of the multi-furrow stump-jump plough, followed later by the mallee roller. These two advances engendered a revolutionary change, by reducing costs and greatly facilitating the task of reclamation. Simultaneously with these developments, the railway network was carried further to the north, and considerable expansion followed in the most northerly counties. Figure 4 shows the distribution in 1884, and indicates clearly the extension in counties Blachford, Hanson, Newcastle, Granville, Frome, and Dalhousie, and the mallee districts of counties Daly and Fergusson, the area of crop sown in regions with a mean seasonal rainfall of less than 10 in. now assuming much larger proportions.

Toward the close of this period, two further, all-important, advances were made:

1. The selection, in 1881, of a variety known as Ward's Prolific, which was fairly resistant to rust, and yielded well under adverse conditions; it formed a base from which other selections were made, and constituted a major turning-point in varietal history.
2. During 1882-85, Custance demonstrated in the field at Roseworthy Agricultural College the value of superphosphate.

(b) 1886-1908

Throughout this interval, the total area remained approximately constant, but the distribution of the crop was altered radically. In the first 10 years further expansion occurred in the northern districts, on Eyre Peninsula, and in counties Burra, Eyre, Sturt, and Albert. These increases were compensated by a reduction in acreage on Yorke Peninsula and in regions of higher rainfall to the north of Adelaide. The distribution in 1896 is given by Richardson (1936). The closing years brought further changes. Much of the far northern section proved unsuitable because of the limited and erratic rainfall, and a marked recession followed,



Fig. 4



Fig. 5

the northern boundary being withdrawn except for isolated localities. At the same time further settlement occurred on Eyre Peninsula in the vicinity of ports, and cropping was again intensified on Yorke Peninsula and in counties Daly, Gawler, and Light. Figure 5, which gives the distribution in 1908, shows also the developments on mallee land near the River Murray in counties Albert and Alfred, and the extension in counties Buckleuch and Chandos following construction of a railway through the latter region.

Finally, from 1900 onward, mean yields increased, thus giving the first tangible results of Lowrie's* efforts to popularize the use of superphosphate.

(c) 1909-32

This period was marked by further withdrawals from the northern counties, but its outstanding feature is the second great surge of development, culminating in the peak of over 4 million acres during 1930-32 inclusive. This enormous expansion followed first from the extension of the railway system, during 1906-19, into the mallee areas of counties Albert and Alfred, and of Eyre Peninsula, and later from the stimulus given by the high prices offered for wheat, and repatriation policies following the 1914-18 war. The average distribution for the years 1924-5-6 is given by Richardson (*loc. cit.*), and that for 1930 in Figure 6.

Other important advances were a general adoption of the practice of applying superphosphate to the crop, the almost universal use of improved varieties that had been developed in the breeding programmes conducted in various parts of the Commonwealth, and the appearance of a close association between sheep- and wheat-farming.

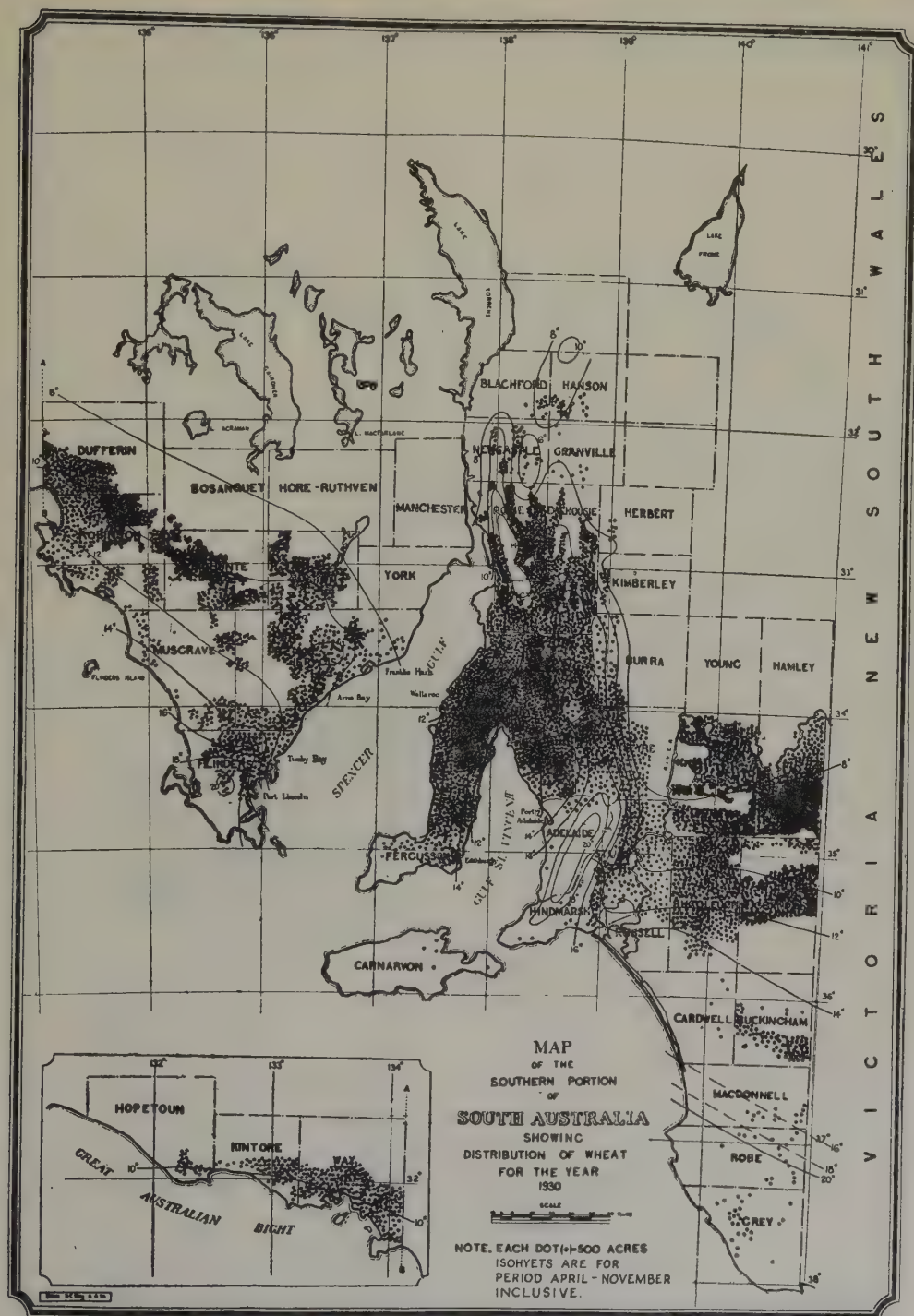
(d) 1933-41

A complete reversal of the trend in acreage characterizes this short sequence of years, and by 1941 the total area in the State had fallen to just over 2¼ million acres, the value attained about 1920. The rapidity with which this major readjustment occurred was due to the impact on the industry of the economic crisis of 1930 following immediately in the wake of four severe droughts during 1926-29. The balance was loaded most heavily against the wheat-growers of the marginal and submarginal regions, and comparison of the distribution in 1941 (Fig. 7) with that of 1930 demonstrates that by far the greater proportion of the reduction was made in the most recently settled tracts of mallee land. Actually, the exigencies of the economic situation only accelerated this reduction, for, as will appear, it was inevitable that the margin of cultivation would be withdrawn in these areas, just as it was in the most northerly parts after the expansive and optimistic settlement of the years 1880-96. This observation may even be extended. In the light of experience and from a consideration of the facts which have become available since 1941, it seems that additional large-scale adjustments should be made.

II. ORIGIN AND SCOPE OF THE INVESTIGATION

The problems associated with settlement of the marginal agricultural areas, which for a long time had been exercising the minds of governmental authorities.

* W. Lowrie succeeded J. D. Custance as Principal of Roseworthy Agricultural College.



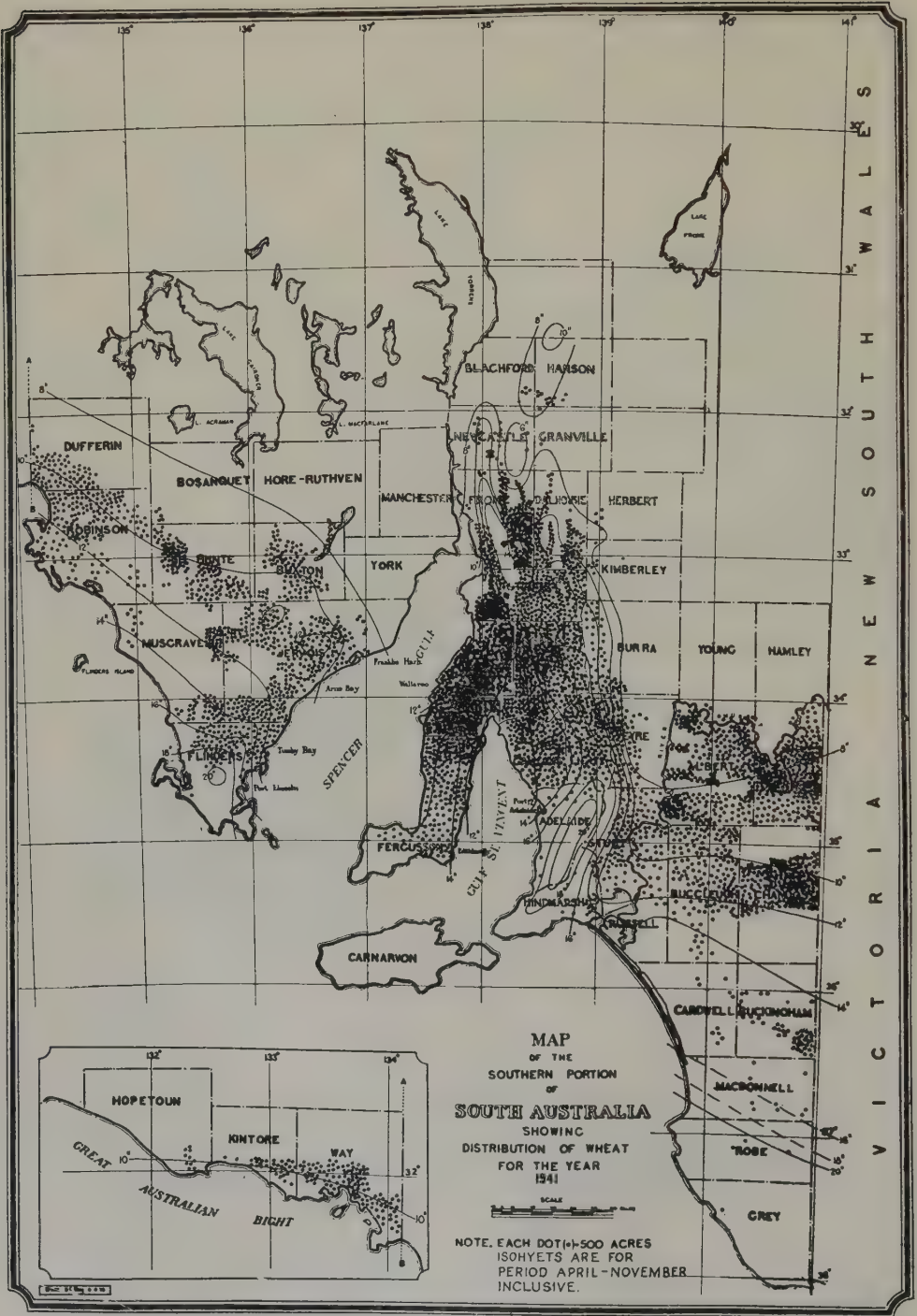


Fig. 7

were brought suddenly into focus by the economic situation, and several enquiries were instituted to study the position of the wheat industry in South Australia. The Agricultural Settlement Committee (1931) reported to the Government on agricultural policy, settlement, and development, and discussed *inter alia* the expansion and disabilities of the industry, the problems of drought relief, and measures for increasing production. The marginal areas were subjected to a particularly close examination, and two papers by Perkins (1934, 1936) again gave them prominence. In the second paper, Perkins segregated the profitable from unprofitable wheat-growing areas, using as a criterion a minimum mean yield of 6 bushels per acre for the 20 years 1915-34 inclusive, and made further constructive suggestions for rehabilitating and stabilizing agriculture in the districts he had designated as unprofitable and unsuitable for the production of wheat as the major crop.

In 1934, the Commonwealth set up a Royal Commission to investigate the economic position of the industry for the whole country. The Commission's second report (1935) covered the wide range of problems confronting the industry, and submitted detailed information relating to costs of production in various districts.

Another step was taken locally in 1939. The Marginal Lands Committee (1939) was formed to consider again the situation as it pertained to South Australia. This Committee defined marginal lands as "areas which have been subdivided into blocks intended principally for wheat-growing and which have been utilized mainly for that purpose, but owing to the combination of an inadequate rainfall and unsuitable land have proved to be unsuitable for wheat as a major operation," and surveyed the wheat belt of the State to determine the areas within the ambit of the problem and the number of settlers involved. The Committee found that a primary cause of failure was the small size of holding, which prevented the grower from carrying sufficient sheep, and necessitated frequent cropping with wheat to maintain income. The solution suggested was to increase the size of holding so that more sheep could be carried, in association with a longer rotation such as fallow, wheat, oats, and pasture for two or more years according to soil type and rainfall, the length of the rotation being largely determined by the rapidity of deterioration of the pasture.

At the same time, an independent and more detailed examination of the available data by the author indicated that the yields in these regions were declining, and an investigation was begun to determine the trends, with the objectives of using them to delineate the areas concerned and for correlation with relevant concomitant observations. In 1940, the remainder of the wheat belt was included, so that all the advantages of contrast could be gained from a comparison with the trends of yield from districts favoured with a higher and more reliable rainfall, the 25-year period 1913-37 inclusive being chosen for examination since at that time it gave the longest standard sequence of yields over the most extensive range of soil types and climatic conditions. The results of this investigation were communicated privately to the Rural Reconstruction Commission in 1944.

Subsequently, when more precise information on the major soil types became available, the scope of the enquiry was widened further by extending the analysis to a longer series of records.

III. DATA AND ANALYTICAL METHOD

(a) *Yield Data*

The smallest territorial unit for which consecutive yield data are available is the hundred, the mean area of which in South Australia is approximately 76,000 acres, or 118 square miles. This unit was adopted, and the yields, expressed as mean yields in bushels per acre *sown for grain*, were extracted from the South Australian Statistical Register for all hundreds with an average area of not less than 1,000 acres under crop. The yields used in compiling the records are not estimates, but are derived from returns submitted by the growers. Errors in the figures quoted (mainly printing errors) were eliminated by checking the means against total production and acreage.

In all, some 296 hundreds have been examined, and their distribution among the regional divisions of the State as given by the Regional Planning Committee (1946), is as follows:

Nuyts	48	Light	30	Flinders	42
Yorke	21	Sturt	12	Eyre	58
Pyap	19	Goyder	37	Fleurieu	1
Pinnaroo	24	Tatiara	3	Adelaide	1

In view of the possibility of future planning requirements, this method of reference was chosen rather than the older statistical subdivisions; an additional reason is provided by the fact that it has already been used in the report of the Pastoral and Marginal Agricultural Areas Inquiry Committee (1948). Figures 8 and 12 show the hundreds in relation to the regional boundaries.

In districts where cropping began prior to 1896, the analysis was confined to the period 1896-1941 inclusive, and where it commenced in 1896 or later only those hundreds which, by 1941, had a record of 20 years or more were selected. These limitations were imposed for three reasons:

1. All records of yield prior to 1896 are incomplete owing to the occurrence of two gaps, totalling 7 years, when no returns were taken, during 1885-88 and 1893-95, and sections of the records which exist do not give yields in sufficient detail for the purposes of the analysis.
2. In 1942, the Commonwealth Government, under war-time legislation, restricted the acreage to be sown to wheat.
3. The minimum of 20 years was chosen so that the trend in yield and the effects of seasonal rainfall could be accurately assessed.

(b) *Rainfall Data*

The finest subdivision of the year for which rainfall data are available in a form convenient for immediate use, is the calendar month. This unit was taken, and the data supplied by the Commonwealth Meteorological Bureau.

As so much was contingent upon making proper allowance for seasonal variations in yield, it was necessary to select the rainfall stations carefully. Owing to the circumstances that the area of each hundred is comparatively large, and that, in many instances, particularly those hundreds on the slopes of the Mt. Lofty and Flinders Ranges, there are considerable changes in altitude, the choice of rainfall stations would have been very difficult and of doubtful value, had recourse not been taken to two subsidiary sets of information:

1. A map showing the location of the area under crop in each hundred.
2. A map of isohyets of April-November rainfall.

On the whole, the rainfall records were remarkably complete, but there were, of course, the inevitable breaks in continuity. These were made good by substituting data from neighbouring stations, selected on the basis of the isohyets and the location of the crop. In the majority of hundreds, only one rainfall record was used, particularly in Nuyts, Eyre, Pyap, and Pinnaroo, where rainfall changes comparatively slowly with position; occasionally there were two, and more rarely three. In all, some 261 records were employed to eliminate the effects of seasonal rainfall.

(c) *Analytical Method*

In the examination of trends during the period 1913-37 the rainfall variates were determined by the following considerations.

(i) *The Nature and Paucity of the Yield and Rainfall Data.*—Since the yields in the greater proportion of hundreds were derived from very extensive areas, and the sequence was confined to a maximum of 25 years, these facts, coupled with the low density of rainfall stations and the enforced decision to employ monthly rainfall, precluded the possibility of making any detailed analysis of the rainfall. At the same time, however, it was recognized that some allowance would have to be made for the differential effects of rains in the several parts of the season.

(ii) *The Average Distribution of Seasonal Rainfall.*—Over the whole wheat belt the distribution of rainfall throughout the season is nearly symmetrical about a date in the interval mid-June to mid-July.

(iii) *The Form of the Regression Function.*—A previous analysis of yield data from Roseworthy College had shown that the regression of yield on rainfall, when expressed as a function of time, assumed the mathematically simple parabolic form, with a maximum in July. It was reasonable to assume that this form of relationship would be typical of most of the wheat-growing areas.*

(iv) *The Period during which Rainfall is Effective.*—It was shown by Trumble (1937) that the outer limits of the area at that time devoted to the cultivation of wheat corresponded roughly with the isochrone giving a season of five months' duration in which rainfall is effective, the average calendar interval being May-September. At the other extreme, the season ran to 7½ months in several isolated localities. The greatest proportion of the wheat belt, however, was situated in the zone between the 5- and

* This assumption will be substantiated in a paper in course of preparation.

7-month isochrones. In seeking a standard suitable over the whole wheat belt, April-November inclusive was chosen, as it gave a reasonable period prior to seeding in every district, and extended to harvest in all but a few places.

After taking account of these points, it appeared that the simplest adequate expressions which would effectively allow for variations in seasonal conditions would be the rains of the following subdivisions:

April and May
June, July, and August
September and October
November,

and these were taken as four rainfall variates.

Similar considerations apply in the analysis of the longer series of records, but the procedure has been modified by omitting November rainfall, since an exhaustive examination of the regressions of yield on this variate which had been obtained previously, showed that very little would be gained by its inclusion. With regard to the present investigation, the partial regressions on the remaining rainfall variates are by-products of the analysis, and their consideration has been relegated to another paper.

It is possible that rainfall of the preceding season and of the period December-March immediately prior to seeding, are correlated with yield, but such effects are also small when compared with those of the current season's rainfall to the end of October, and consequently they have been ignored. In any case, the crop records are not of sufficient length to account for them adequately.

At this juncture, it is important to mention two additional points. In the first place, only one meteorological element has been used to characterize the season, so that, in correlating it with yield, the regression obtained is the resultant of a number of components, one, the effect of the rainfall *per se*, and the remainder due to the direct and indirect effects of all other elements associated with the rainfall occurring in the several subdivisions under consideration. In the second place, it is obvious that definite optimal conditions must apply in various parts of the season, and the effects of the rainfall at all times are not strictly independent and additive. In the present enquiry, the quadratic terms, corresponding to such factors, have also been omitted, since in comparison with the linear terms they are of much less quantitative importance.

Four different functions of time have been employed to represent the temporal trends of yield. If y denotes yield, x_1, x_2, x_3 the rainfall variates in the order previously given, x_4 and x_5 time and its square, the four types of multiple regression were:

$$Y = \bar{y} + b_1(x_1 - \bar{x}_1) + b_2(x_2 - \bar{x}_2) + b_3(x_3 - \bar{x}_3) + b_4(x_4 - \bar{x}_4) \dots (1)$$

$$Y = \bar{y} + b_1(x_1 - \bar{x}_1) + b_2(x_2 - \bar{x}_2) + b_3(x_3 - \bar{x}_3) + b_4(x_4 - \bar{x}_4) + b_5(x_5 - \bar{x}_5) \dots (2)$$

$$Y = b_1(x_1 - \bar{x}_1) + b_2(x_2 - \bar{x}_2) + b_3(x_3 - \bar{x}_3) + b_4x_4^{b_5} \dots (3)$$

$$Y = b_1(x_1 - \bar{x}_1) + b_2(x_2 - \bar{x}_2) + b_3(x_3 - \bar{x}_3) + x_4/(b_4 + b_5x_4), \dots (4)$$

the bar over a symbol designating the arithmetic mean.

The coefficients were determined by the method of maximal likelihood (Fisher 1922), which, in the first two expressions, reduces directly to the standard technique of partial regression. In types (3) and (4) the logarithm of the likelihood is proportional to

$$L = S(y - Y)^2,$$

where the summation is taken over the n observations in the sample, and the equations which must be satisfied on maximizing L for variations in the coefficients are

$$\frac{\partial L}{\partial b_i} = 0, \quad i = 1, 2, \dots, 5.$$

Since these equations are non-linear in the parameters, the ordinary procedure of least squares is not directly applicable, but the solution may be obtained by iteration, taking as a starting-point any conveniently calculable approximate values.

If \widehat{b}_i and b'_i denote the maximal likelihood estimate and its approximation respectively, and

$$\hat{b}_i = b'_i + a_i,$$

the corrections to be applied are the solution of the equations

$$\left. \begin{aligned} \frac{\partial L}{\partial b_1} &= \frac{\partial L}{\partial b_1} + a_1 \frac{\partial^2 L}{\partial b_1^2} + a_2 \frac{\partial^2 L}{\partial b_1 \partial b_2} + \dots + a_5 \frac{\partial^2 L}{\partial b_1 \partial b_5} = 0 \\ \frac{\partial L}{\partial b_2} &= \frac{\partial L}{\partial b_2} + a_1 \frac{\partial^2 L}{\partial b_1 \partial b_2} + a_2 \frac{\partial^2 L}{\partial b_2^2} + \dots + a_5 \frac{\partial^2 L}{\partial b_2 \partial b_5} = 0 \\ &\vdots \\ \frac{\partial L}{\partial b_5} &= \frac{\partial L}{\partial b_5} + a_1 \frac{\partial^2 L}{\partial b_1 \partial b_5} + a_2 \frac{\partial^2 L}{\partial b_2 \partial b_5} + \dots + a_5 \frac{\partial^2 L}{\partial b_5^2} = 0 \end{aligned} \right\} \dots (5)$$

where in the set of derivatives on the extreme left, b_i is replaced by \hat{b}_i after differentiation, and in the remainder by b'_i .

The solution is best obtained by inverting the matrix of second derivatives in equations (5), and if

$$[c_{ij}] \quad i, j = 1, 2, \dots, 5$$

denotes the inverse matrix, the a_i are given by

$$a_i = S_j c_{ij} \left(-\frac{\partial L}{\partial b_i} \right)_{b_i = b'_i} \quad i, = 1, 2, \dots, 5.$$

These corrections are added to the approximate values, and the whole process repeated if necessary.

The residual variance, s^2 , is determined from the relation

$$s^2 = \frac{1}{(n-5)} S(y - Y)^2$$

since 5 adjustable parameters have been calculated from the data, and finally, the variance-covariance matrix of the \hat{b}_i is computed by multiplying the elements

of the inverse of the matrix

$$\left[\begin{pmatrix} \frac{\partial^2 L}{\partial b_i \partial b_j} \end{pmatrix} \begin{matrix} b_i = \hat{b}_i \\ b_j = \hat{b}_j \end{matrix} \right] \quad i, j = 1, 2, \dots, 5$$

by s^2 .

The arithmetical detail of the process is illustrated with the calculations for the hundred of Crystal Brook, to the yields of which a multiple regression of type (3) was fitted. The steps in order are as follows:

1. Approximate values b'_1 (1.5208), b'_2 (2.0273), and b'_3 (0.6894) are found by fitting a multiple regression with x_1 , x_2 , x_3 , and x_4 as independent variates, and the yields corrected for variations in seasonal rainfall.

2. Approximate values b'_4 (5.140) and b'_5 (0.2922) are then determined by taking the regression of log (corrected yield) on log (time).

3. After substituting these approximations, the numerical values of the elements in the matrix of coefficients and the quantities on the right-hand sides of equations (5) reduce to

		$\frac{\partial^2 L}{\partial b_i \partial b_j}$			$\frac{\partial L}{\partial b_j}$
76.09	11.27	-1.34	-2.33	-88.21	-10.2270
11.27	158.18	40.32	-2.71	-104.74	-12.7233
-1.34	40.32	105.02	3.79	87.00	-18.3782
-2.33	-2.71	3.79	276.54	4482.12	26.1761
-88.21	-104.74	87.00	4482.12	76774.51	425.7731

and the inverse of the matrix of second order derivatives is

		c_{ij}		
13.40377	-1.03221	0.52849	-2.25917	0.14528
-1.03221	7.15029	-2.78873	-1.68294	0.10998
0.52849	-2.78873	10.62995	1.45959	-0.10045
-2.25917	-1.68294	1.45959	68.17862	-3.98684
0.14528	0.10998	-0.10045	-3.98684	0.24621

each element being multiplied by 10^{-8} .

The correction term a_1 , for example, is then

$$\{ (-13.40377 \times 10.2270) + (1.03221 \times 12.7233) - (0.52849 \times 18.3782) - (2.25917 \times 26.1761) + (0.14528 \times 425.7731) \} \times 10^{-8} = -0.1309.$$

The corrections and second approximations are

a_1	a_2	a_3	a_4	a_5
-0.1309	-0.0264	-0.1698	0.1049	-0.0006
b_1	b_2	b_3	b_4	b_5
1.3899	2.0009	0.5196	5.2449	0.2916

4. Repetition of the process using the second approximations gives the quantities

		$\frac{\partial^2 L}{\partial b_i \partial b_j}$				$-\frac{\partial L}{\partial b_j}$
76.09	11.27	-1.34	-2.32	-89.69		-0.0049
11.27	158.18	40.32	-2.70	-106.50		0.0060
-1.34	40.32	105.02	3.78	88.70		-0.0018
-2.32	-2.70	3.78	275.48	4640.48		0.2139
-89.69	-106.50	88.70	4640.48	81162.64		-4.8716

with inverse matrix

		c_{ij}				
13.43794	-1.00674	0.50627	-3.31375	0.20244	} x 10 ⁻⁸	
-1.00674	7.16927	-2.80529	-2.46979	0.15257		
0.50627	-2.80529	10.64437	2.14881	-0.13761		
-3.31375	-2.46979	2.14881	100.43595	-5.75168		
0.20244	0.15257	-0.13761	-5.75168	0.34175		

yielding the following second corrections and third approximations:

a_1	a_2	a_3	a_4	a_5	} \dots (6)
-0.0018	-0.0012	0.0011	0.0495	-0.0029	
b_1	b_2	b_3	b_4	b_5	
1.3881	1.9997	0.5207	5.2944	0.2887	

At this point the working could have been terminated, as the solution is sufficiently accurate, but the calculations are here taken through a further stage, using the third approximations, to show that the third corrections are negligible. The numerical values are as follows:

		$\frac{\partial^2 L}{\partial b_i \partial b_j}$				$-\frac{\partial L}{\partial b_j}$
76.09	11.27	-1.34	-2.28	-89.01		0.0501
11.27	158.18	40.32	-2.64	-105.67		-0.0122
-1.34	40.32	105.02	3.73	89.14		0.0062
-2.28	-2.64	3.73	270.40	4593.47		0.0804
-89.01	-105.67	89.14	4593.47	81049.55		1.4668

		c_{ij}				
13.43509	-1.00801	0.50610	-3.28818	0.19924	} x 10 ⁻⁸ \dots (7)	
-1.00801	7.16898	-2.80602	-2.47674	0.15169		
0.50610	-2.80602	10.64593	2.19272	-0.13908		
-3.28818	-2.47674	2.19272	101.36102	-5.75387		
0.19924	0.15169	-0.13908	-5.75387	0.33901		

a_1	a_2	a_3	a_4	a_5
0.0007	-0.0001	0.0001	-0.0004	0.0000
b_1	b_2	b_3	b_4	b_5
1.3888	1.9996	0.5208	5.2940	0.2887

5. Taking the values in (6) as the solution, the residual sum of squares is 275.07, with 41 degrees of freedom ($n = 46$), giving a residual variance of 6.71,

from which the variances of the b_i are obtained by multiplying in turn by the diagonal elements of the matrix (7). The standard deviations and five values of t (Fisher 1946) in order are

Standard deviation	0.300	0.219	0.267	0.825	0.048
t	4.63	9.13	1.95	6.42	6.02

(41 degrees of freedom)

so that b_3 is the only insignificant coefficient.

After fitting the multiple regression (3), the sum of squares due to the regression formula can be very conveniently derived from the expression

$$b_1Sy(x_1 - \bar{x}_1) + b_2Sy(x_2 - \bar{x}_2) + b_3Sy(x_3 - \bar{x}_3) + b_4Sx_4^{b_5} \dots \dots (8)$$

but for regressions of type (4) the formula cannot be written so simply, and takes the form

$$\begin{aligned} & b_1^2S(x_1 - \bar{x}_1)^2 + b_2^2S(x_2 - \bar{x}_2)^2 + b_3^2S(x_3 - \bar{x}_3)^2 + 2b_1b_2S(x_1 - \bar{x}_1)(x_2 - \bar{x}_2) \\ & + 2b_1b_3S(x_1 - \bar{x}_1)(x_3 - \bar{x}_3) + 2b_2b_3S(x_2 - \bar{x}_2)(x_3 - \bar{x}_3) \\ & + 2Sy\left(\frac{x_4}{b_4 + b_5x_4}\right) - S\left(\frac{x_4}{b_4 + b_5x_4}\right)^2 \dots \dots \dots (9) \end{aligned}$$

For all cases in which regressions of types (1) and (2) have been used, the multiple correlation coefficient has been determined, and thence the percentage of variance of yield, A , ascribable to the average effects of the rainfall variates and time, from the relation

$$A = 1 - \frac{(n-1)}{(n-p-1)} (1-R^2) \dots \dots \dots (10)$$

where R is the multiple correlation coefficient, n is the number of observations in the sample, and p the number of independent variates (Fisher 1924).

The sums of squares due to the regression formulae of types (3) and (4) contain the ordinary correction for the mean, $n\bar{y}^2$, and if this is deducted from both sums an index of multiple correlation may be defined, by analogy with the multiple correlation coefficient, as the ratio

$$R^2_i = \frac{SY^2 - 2SyY - n\bar{y}^2}{S(y - \bar{y})^2} \dots \dots (11)$$

and the percentage of variance, corrected for positive bias in R^2_i , will be correspondingly

$$A_i = 1 - \frac{(n-1)}{(n-p-1)} (1-R^2_i), \dots \dots \dots (12)$$

p now designating the number of coefficients in the regression formula after deducting one for the mean. R_i and A_i have been determined for all hundreds in which they are appropriate.

IV. SOIL GROUPS OF THE WHEAT BELT

The geographical distribution of the soil groups in relation to the isohyets of seasonal rainfall and the boundaries of the hundreds, is illustrated in Figure 8. This map summarizes the best information available at the present time. It must be realized that the allocations have been made on a rather broad basis; within the boundaries as mapped, variations of type exist owing to geological

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Page 100, equation (11):

$$\text{For } R^2, = \frac{SY^2 - 2SyY - n\bar{y}^2}{S(y - \bar{y})^2} \text{ read } R^2, = \frac{2SyY - SY^2 - n\bar{y}^2}{S(y - \bar{y})^2}$$



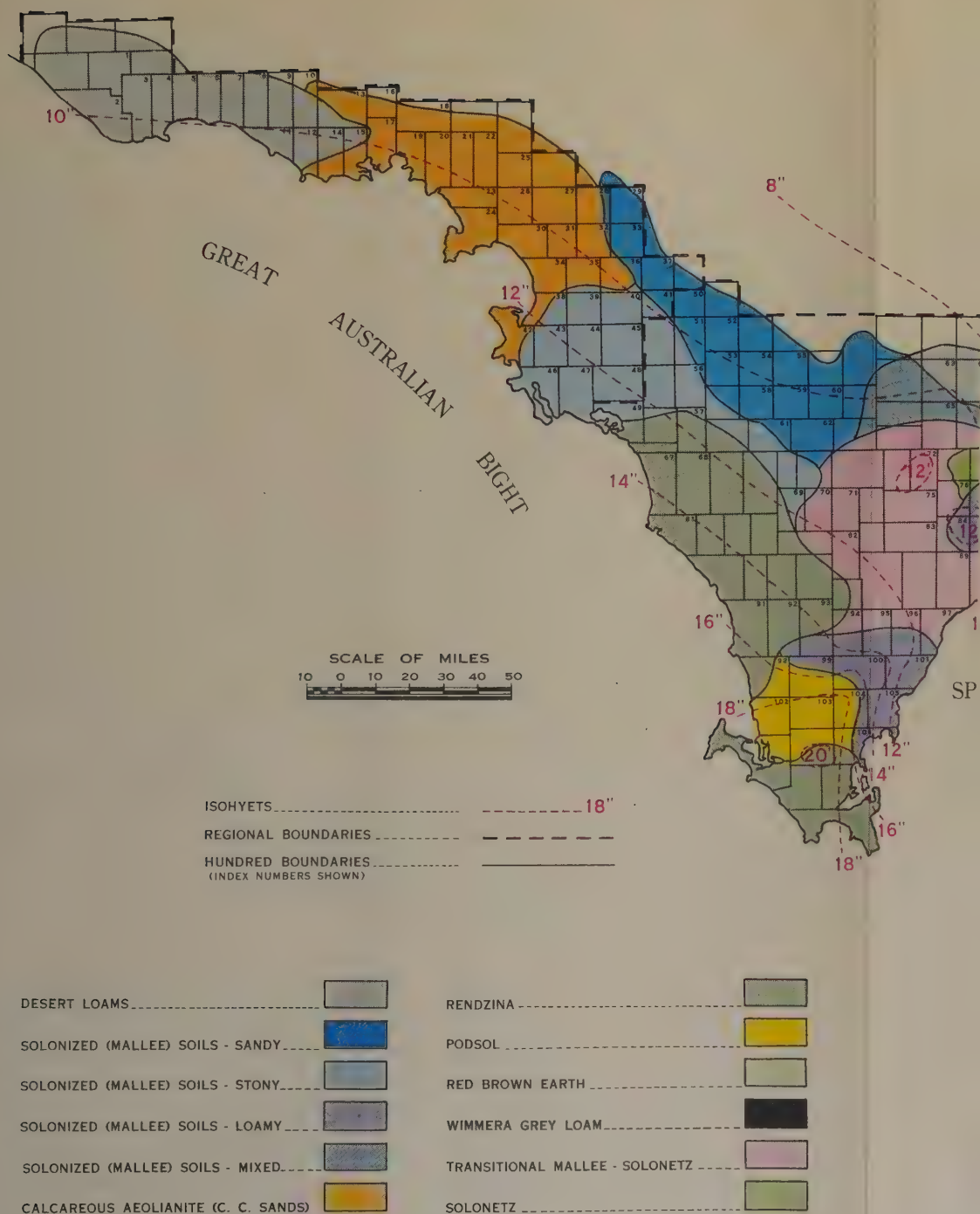
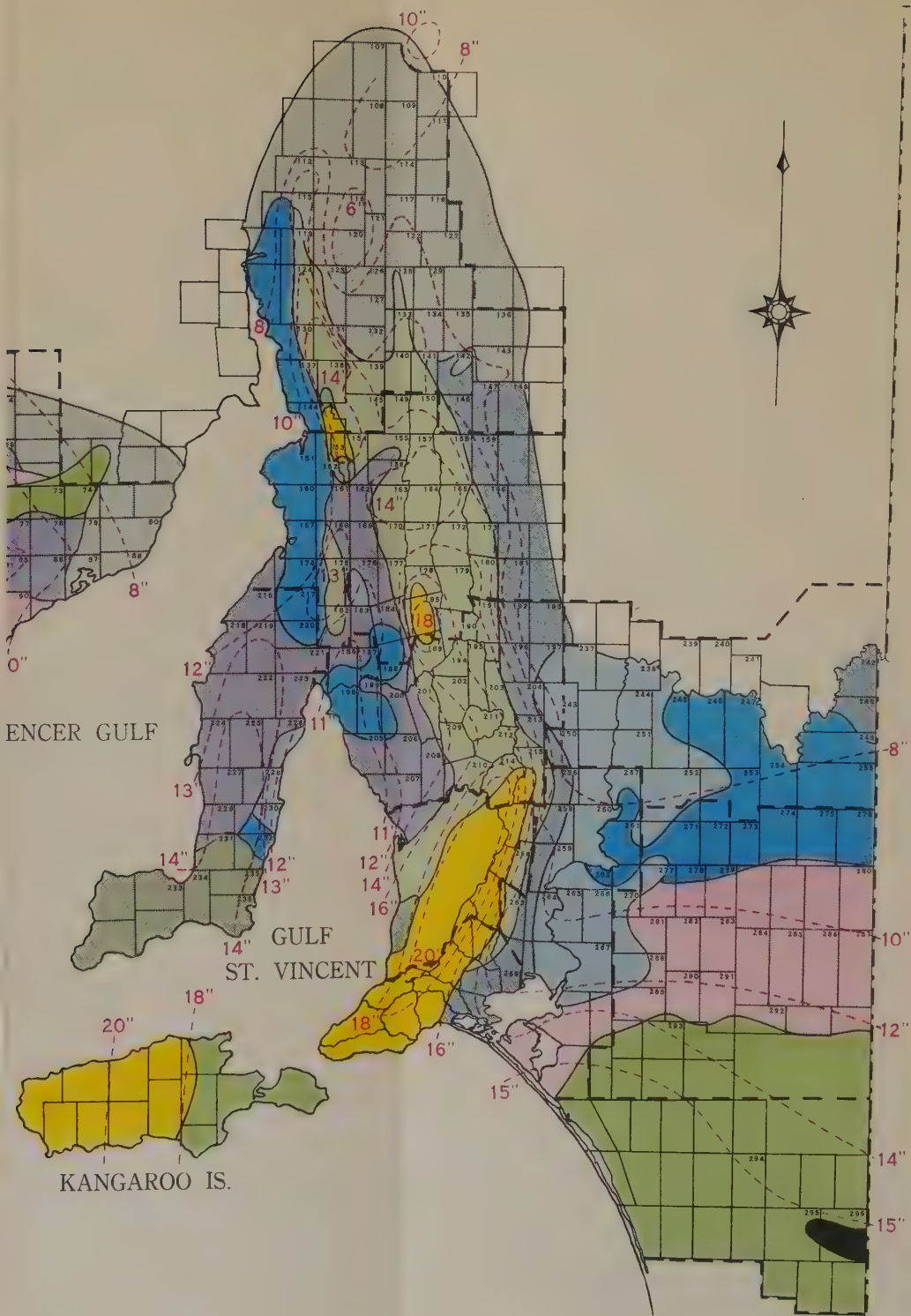


Fig. 8. Major Soils Zones in the Wheat Belt of South Australia (drawn from)



m data supplied by the Division of Soils, C. S. I. R., and R. I. Herriot).

and climatic differentiation, and consequently, if the grouping is applied in fine detail it is liable to be misleading.

The descriptions of the soil groups are as follows:

(i) *Podsols*.—These are leached soils with a grey surface and generally a yellow or mottled clay subsoil. The surface soil is commonly of light texture, and acid in reaction; lime is absent from the profile except where the soil is residual on lime-bearing rocks, and even under these conditions it occurs only in the lowest horizon. The group as mapped includes the lateritic podsols of Kangaroo Island, the Mt. Lofty Ranges, and southern Eyre Peninsula, where ironstone gravel or massive laterite appears in the profile at or near the surface. Fertility of the podsols is low to moderate, but generally enhanced by humid climatic conditions.

(ii) *Red Brown Earths*.—This group has brown surface soils usually loamy in texture with red or red-brown structured clay subsoils. The surface is neutral to slightly acid in reaction, and lime occurs in the deeper subsoil. These are the most fertile of the soils used extensively for wheat-growing in South Australia, and are favoured by a fairly reliable winter rainfall. In the zone as mapped, there are very restricted occurrences of black earth or chernozem soils, the principal one being at Saddleworth.

(iii) *Desert Loams*.—In these brown soils of the arid areas there is usually a light to moderately heavy surface horizon over a clay subsoil in which occurs an accumulation of lime, and frequently, visible gypsum. The profile is alkaline throughout. The fertility of this group is very low and the various types erode rapidly under cultivation.

As mapped, the group includes:

1. The desert loams, which constitute the major portion of Flinders.
2. The types occurring at the western extremity of the wheat belt in the vicinity of Fowler's Bay. These are grey-brown loamy soils of varying depth which possess a high salinity at or near the surface in many parts, and have affinities to the desert loams.
3. The loamy mallee soils extending in an arc from Kimba to Cowell. These soils occur in a region of low rainfall, and hence are agriculturally similar to the desert loams and have accordingly been included with them.

Both the regions of the true desert loams and red brown earths are characterized by a high proportion of skeletal soils on the hills and ranges, too stony and shallow for cultivation.

(iv) *Rendzinas*.—Black soils, occasionally degraded to grey or grey-brown by saline conditions or leaching, lying residual on soft and/or clayey calcareous parent material. The surface is neutral to slightly alkaline in reaction. The soils are irregular in depth, of variable fertility, and occur over a moderately wide range of climatic conditions.

(v) *Solonized Soils*.—Soils of this type constitute the largest single group in the agricultural regions of South Australia. They are brown in colour and have been solonized. A prominent morphological feature is the large accession

of calcium carbonate which was probably wind-borne.

The main group includes a subgroup originally described as mallee soils, and it is proposed to use this term here, since all occurrences in the wheat belt of the State carry the characteristic mallee vegetation associations (Wood 1937).

Texture is variable, and reaction alkaline. Different regions are dominated by (1) loamy soils; (2) stony soils where the development, exposure, and disintegration of a travertine limestone pan have resulted in a stony surface soil; (3) sandy soils, some of which have arisen by the accumulation of siliceous sands, stripped by aeolian action from the leached surface horizons of adjacent stony areas. Where all three or any two of these types occur together with no one predominant, the soils have been classed and mapped as mixed mallee.

A related class, described by the term calcareous aeolianite, has also been included in this group. These soils are consolidated calcareous sands which have *not* been solonized but in their original state carried mallee (*Eucalyptus oleosa*) interspersed with open spear grass (*Stipa* spp.) plains.

(vi) *Solonetz Soils*.—Former concentrations of salt have affected the profiles of these soils but they now contain relatively small amounts. The subsoil contains a marked proportion of exchangeable sodium. The soils are generally of light texture, particularly in the surface horizon, and in South Australia have developed on modified sand-ridge country. They are neutral to alkaline in the surface, and alkaline in the subsoil, and occur over a moderately wide range of climatic conditions. Fertility varies according to the character of the precursor soil and the stage of solonization, but is principally low.

(vii) *Transitional Mallee-Solonetz Soils*.—In the zone between the recognized brown solonized and solonetz soils, there occurs a substantial area featuring the characteristic mallee physiographic pattern of alternating dunes and flats, the former being solonetz, and the latter typical brown solonized soils; the proportion of solonetz to solonized soil increases on any line approaching the solonetz region. The rainfall of these areas is generally higher than that of the mallee, and cropping is confined mainly to the interdunal spaces.

(viii) *Wimmera Grey Soils*.—The profile, which is alkaline and may be partly solonized, is uniformly heavy, consisting of grey or brown clays, becoming mottled with depth; the grey clays may have a self-mulching surface. Lime and gypsum occur to some degree in the subsoil. The South Australian occurrence of this type is a small intrusion at the western extremity of the fertile soils of the Victorian Wimmera.

V. SUMMARY OF RESULTS AND DISCUSSION

(a) *Accuracy of the Regression Formulae*

The complete tabulation of the results of this investigation has not been included, since it is too extensive for reproduction herein. Copies have, however, been filed, and are available for inspection.*

*At the libraries of the Council for Scientific and Industrial Research, 314 Albert Street, East Melbourne, and its Section of Mathematical Statistics, University of Adelaide.

The statistical significance, with minimum odds of 19:1, has been established for the trends in yield of practically all hundreds except those which have been classified as type III of Figure 11 (*vide infra*), and by appropriately combining the probabilities of both significant and insignificant coefficients, any doubts regarding the few exceptions are removed. Such tests are, however, really not required, since the close similarity of form in the trends of contiguous hundreds provides sufficient proof.

Table 1 sets out the distribution of the coefficients and indices of multiple correlation within arbitrary, but convenient, subdivisions of the range of seasonal rainfall (total of April-November inclusive) and the mean values of the percentage variance, the value 83 per cent., for example, being the mean of the 14 cases in which the coefficient or index was ≥ 0.91 . In 267 hundreds, 50 per cent. or more of the observed variation in yield is expressible in terms of the four types of function employed in the analysis.

TABLE 1
DISTRIBUTION OF COEFFICIENTS AND INDICES OF MULTIPLE CORRELATION
WITHIN ARBITRARY CLASSES OF SEASONAL RAINFALL

Correlation	Rainfall (in.)							Total	Per Cent. Variance (A)
	< 8	8-10	10-12	12-14	14-16	16-18	> 18		
≥ 0.91	7	2	2	3				14	83
0.81-0.90	39	54	44	29	3			169	70
0.71-0.80	9	34	19	9	9	3	1	84	53
0.61-0.70		3	12	4	1	1		21	37
0.51-0.60		2	1	2		1		6	25
0.41-0.50				2				2	14
Total	55	95	78	49	13	5	1	296	

The relation which exists between the measures of correlation and seasonal rainfall reflects largely the strength of the relation between yield and rainfall, since, in the majority of hundreds, the regression of yield on time is the smaller contributor to the total variation ascribable to the regression formula. Considering the results as a whole, it can be claimed that a rainfall record provides a sufficiently accurate index of seasonal conditions in this environment.

Table 2 lists the distribution of the correlations within the several soil types of the wheat belt. As rainfall and soil type are, to some extent, related, these distributions are not completely independent of that in Table 1, but, nevertheless, they do indicate that the claim made above holds also on all principal soils. The grand total of 335 does not agree with that of Table 1 because 39 hundreds, comprised equally of two soil types, have been included under both in preparing the table.

(b) *The Types of Trend Which Occur*

Before proceeding further with the discussion, it will be helpful to amplify certain points mentioned in Section I, and introduce others, so that each individual sequence of yields can be viewed in its proper relation to the full

cropping history of the corresponding district. With this objective, it is convenient to divide the hundreds into two categories: (i) those in which cropping began prior to 1896; and (ii) those in which cropping began during or after 1896.

TABLE 2
DISTRIBUTION OF COEFFICIENTS AND INDICES OF MULTIPLE CORRELATION
WITHIN SOIL TYPES*

Correlation	Soil Type										
	Mallee Types					Desert Loam	Solonetz	Red Brown Earth	Rendzina	Podsol	Total
	Sandy	Stony	Loamy	Mixed	Transitional Calcareous Aeolianite						
≥ 0.91	2	4	3	3		3		2			17
0.81-0.90	39	10	27	19	6	15	28	1	34	6	186
0.71-0.80	16	8	9	5	15	7	15	2	13	2	97
0.61-0.70	2	3	4		9	1	1	1	1	3	26
0.51-0.60		1			2		1			1	6
0.41-0.50			1		1				1		3
Total	59	26	44	27	33	23	48	5	50	12	335

(i) *Hundreds in which Cropping Began prior to 1896.*—By 1896, districts in this class had records of yield varying up to 50 years in length, the duration of record being fairly strongly and positively correlated with seasonal rainfall. Since the land was virgin, yields obtained at the outset were reasonably high, and in some localities were probably maintained for varying short periods. Three principal systems of cropping came into use, which, in order of appearance, were wheat continuously, fallow-wheat, and fallow-wheat-oats, and no returns were made to the soil other than what was introduced in the fallows. These methods inevitably led to depletion of essential chemical elements, and, with phosphorus in particular, to almost complete exhaustion of soil reserves. With the consequent reduction of fertility, yields began declining at rates varying from district to district, depending upon initial fertility, date of settlement, rainfall, intensity of cropping, and other subsidiary factors, of which wind and water erosion were probably the most important. The available evidence indicates that during the last ten years of the century yields in practically all districts had reached a very low level.

During 1896-1910, the use of superphosphate had spread widely, and as is well known, there was an immediate and marked response in yield. The great changes in acreage on Yorke Peninsula and in counties Gawler, Light, and Daly between 1884 and 1908 are, in part, a reflection of these facts.† There seems little doubt that the area of wheat was reduced because yields had fallen to such

† Richardson's illustration of the distribution in 1896 reveals a very considerable reduction in the area of wheat for these parts of the State.

* Wimmera grey loam included with red brown earth.

a low level under the intense cropping (*vide* Figs. 3 and 4) of previous years: the response to superphosphate provided the stimulus for the re-intensification of cropping. But, apart from the increase in the phosphorus status of the soils, the advances made by the introduction of improved varieties and the closer association of sheep- and wheat-farming, the factors that had previously been responsible for the decline in yield were still operating, with the result that the rate of improvement was not generally maintained. In Figure 9, the courses of

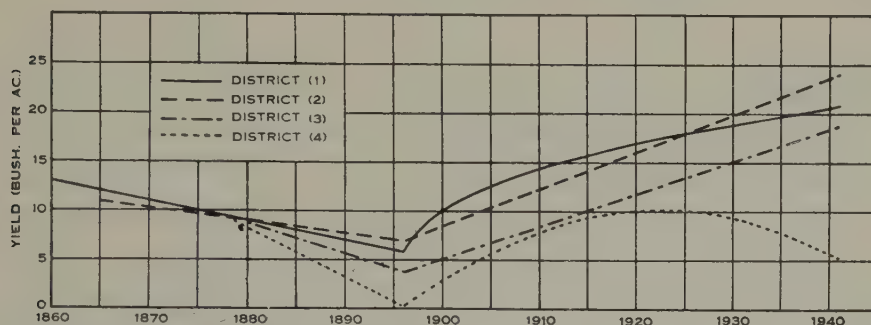


Fig. 9.—Reconstruction of the entire course of yield in four districts opened prior to 1896.

yield in four hundreds have been reconstructed, after making allowances for variations in seasonal rainfall, to illustrate some of these points. The following notes on each district are relevant:

District	Soil Type	Rainfall	Cropping Commenced
(1)	Red brown earth	14-16 in.	1850-1855
(2)	Red brown earth and loamy mallee	14-16 in.	1860-1865
(3)	Loamy mallee	12-14 in.	1880 (exact)
(4)	Mixed mallee	10-12 in.	1878 (exact)

The declines prior to 1896 are represented as linear functions of time, but it is probable that if complete data had been available they would have been found to be exponential. The differences between districts (1) and (2) during 1860-96 are consistent with the fact that the first possessed the more fertile soil, which had been cropped for some ten years longer. Districts (3) and (4) also fall appropriately into sequence according to their rainfall and the natural fertility of the soils. Their initial yields are considerably lower than, and the rates of decline exceed, those of (1) and (2).

After 1896, the response to superphosphate and other factors tending to increase yield, is manifest in the four curves, but their forms vary according to the general conditions in each area. A high rate of increase is present for varying short periods immediately after 1896 in all hundreds opened for settlement before that date, but has only been definitely established for curves similar to that of district (1) and one other type. In such cases the period of cropping before 1896 was generally long and intense. A second important factor which also partly accounts for the difference between districts (1) and (2) is that in the latter

there has been a rapid increase during recent years in the number of stock, mainly sheep, associated with wheat-farming, accompanied by a lengthening of rotations, whereas in the former the general tendency has been to maintain the system based on short-term rotations.

District (4) demonstrates clearly the inability of these particular soils to withstand frequent cropping, since yield eventually declines again notwithstanding the advancements made in the past 40 years.

The six principal types of trend which occur are illustrated in Figure 10.

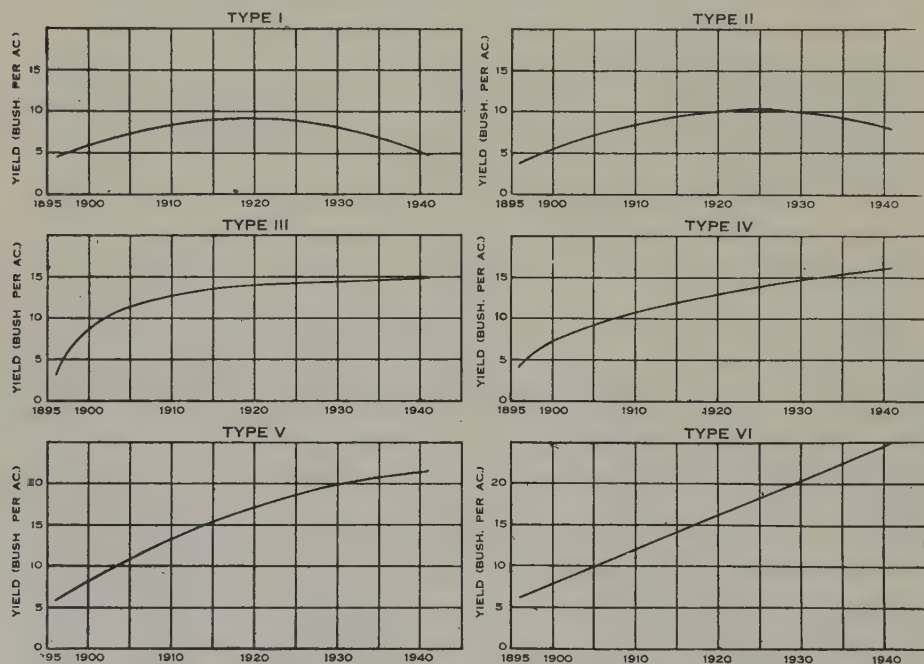


Fig. 10.—The principal types of yield trend in hundreds opened before 1896.

Types I and VI represent the extremes of the range, and there is a gradual transition from type to type within it.

(ii) *Hundreds in which Cropping Began during or after 1896.*—In this category, settlement coincided with the introduction of superphosphate, or occurred at a later date. The hundreds concerned are scattered over a wide range of soil and season, but the bulk of the group is located in the mallee areas of Nuyts, Eyre, Pinnaroo, and Pyap. In these regions, cropping methods in the early stages are exploitative through circumstances incidental to pioneering on land carrying mallee vegetation. Mallee stumps remaining in the ground after the first clearing repeatedly send out new growth, and the settler crops continuously for some years, burning the stubbles to destroy the shoots. The situation is also made more acute since the growers have only limited capital and credit resources, and this provides an additional reason for adopting such methods in an endeavour to improve the income of the first few years. During

this phase, which takes on the average about five years to complete, the principal factor limiting yield, apart from rainfall, is probably a deficiency of available nitrogen. In the course of time, however, as fallowing and other measures for increasing production are introduced, yields would be expected to improve, but the subsequent course would vary according to soil, rainfall, and the practices adopted. The types which occur are illustrated in Figure 11. The range is not

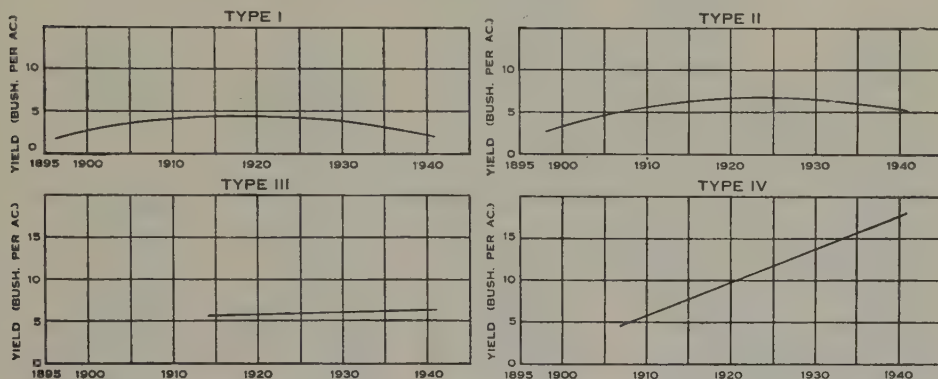


Fig. 11.—The principal types of yield trend in hundreds opened in 1896 or later.

as great as that of Figure 10, since the situation is not complicated by the effects of cropping prior to the advent of superphosphate. Type III applies to hundreds which have only been settled recently, the records being so short that the position with respect to trend is still indeterminate. The positive regressions on time are all statistically insignificant, and from this restricted point of view the sequences of yields are in accord with the hypothesis that the trends are zero, but consideration of all relevant information indicates clearly that hundreds classed in this group are repeating, some 10-20 years later, the first stage of either curve I or curve II. In this sense, the type III curve may be regarded as a subtype of I or II. Included in this group also are several cases in which the trend is practically zero. Hundreds with trends of type IV are easily distinguished, since they are almost entirely confined to two soil classes, and the regressions are strongly significant and of much greater magnitude than those of type III.

In general, both sequences of types are intimately related to improvement in the class of soil and in particular are arranged in order of increasing abundance of nitrogen; this point is elaborated further at various stages below in this section.

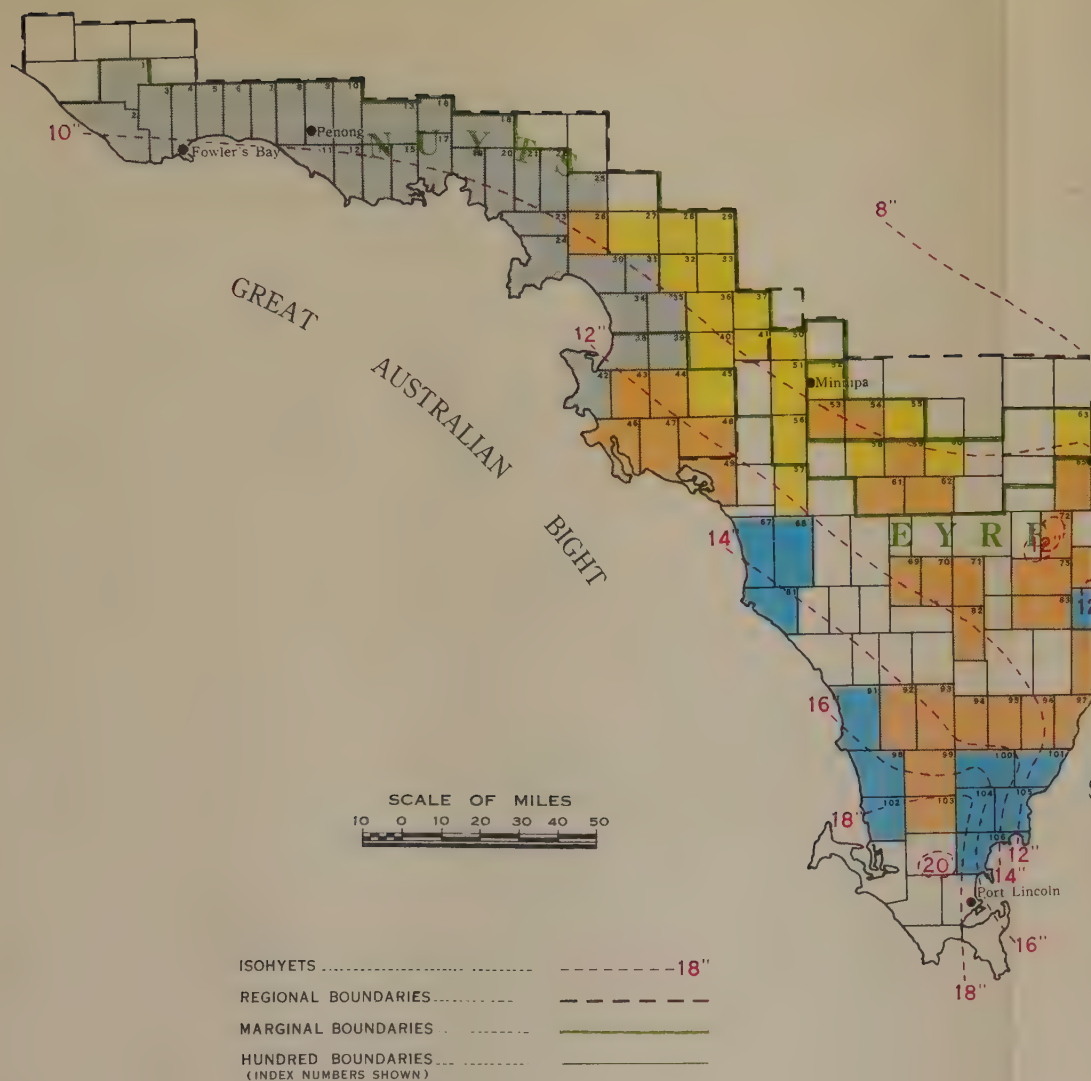
(c) Survey of the Trends and Their Distribution

Figure 12 depicts the geographical distribution of the trends. Diagrammatic representation has been simplified by assigning a specified type of trend to the whole of each hundred to avoid the complication that would inevitably ensue on a map of this scale if the actual location of the crop were given in each. Throughout the remainder of this section, frequent reference will be made to the curves of Figures 10 and 11, and to save repetition they will be quoted as, for example, I(10), III(11).

INDEX OF HUNDREDS

<i>Nuyts*</i>	<i>Eyre (cont.)</i>	<i>Flinders (cont.)</i>	<i>Goyder (cont.)</i>	<i>Pyap (cont.)</i>
1. Miller	64. Moseley	126. Coonatto	185. Clare	243. Brownlow
2. Wookata	65. Solomon	127. Pinda		244. Murbko
3. Sturdee	66. Kelly	128. Eurlia	<i>Light</i>	245. Waikerie
4. Caldwell	67. Colton	129. Oladdie	186. Goyder	246. Holder
5. Nash	68. Talia	130. Winninowie	187. Stow	247. Moorook
6. Magarey	69. Barwell	131. Gregory	188. Hall	248. Paringa
7. Giles	70. McLachlan	132. Willowie	189. Upper	249. Gordon
8. Cohen	71. Palkagee	133. Coomooroo	Wakefield	250. Anna
9. Burgoyne	72. Pascoe	134. Walloway	190. Stanley	251. Paisley
10. Bagster	73. James	135. Erskine	191. Apoinga	252. Bakara
11. Kevin	74. Glynn	136. Cavenagh	192. Bright	253. Mantung
12. Keith	75. Smeaton	137. Baroota	193. Bunday	254. Pyap
13. Catt	76. Campoona	138. Wongyarra	194. Saddleworth	255. Bookpurnong
14. Horn	77. Mangalo	139. Booleroo	195. Waterloo	
15. Bartlett	78. Miltalie	140. Pekina	196. English	<i>Sturt</i>
16. O'Loughlin	79. Minbrie	141. Black Rock	197. Bower	256. Bagot
17. Moule	80. Warren	Plain	198. Inkerman	257. Nildottie
18. Goode	81. Ward		199. Balaklava	258. Angas
19. Bonython	82. Tooligie	142. Morgan	200. Dalkey	259. Finniss
20. Wandana	83. Rudall	143. Coglein	201. Alma	260. Ridley
21. Chillundie	84. Yadnarie	144. Telowie	202. Gilbert	261. Forster
22. Guthrie	85. Mann	145. Appila	203. Julia Creek	262. Younghusband
23. Blacker	86. Hawker	146. Yongala	204. Neales	263. Monarto
24. Wallanippie	87. Playford	147. Gumbowie	205. Dublin	264. Mobilong
25. Hague	88. Wilton	148. Parnaroo	206. Grace	265. Burdett
26. Carawa	89. Roberts		207. Port Gawler	266. Ettrick
27. Petina	90. Boothby	<i>Goyder</i>	208. Mudlawirra	267. Seymour
28. Wallala	91. Kiana	149. Tarcowie	209. Light	
29. Koolgera	92. Mitchell	150. Mannanarie	210. Nuriootpa	<i>Adelaide</i>
30. Haslam	93. Shannon	151. Pirie	211. Kapunda	268. Tungkillo
31. Perlubie	94. Brooker	152. Napperby	212. Belvidere	
32. Walpuppie	95. Moody	153. Howe	213. Dutton	<i>Flenzieu</i>
33. Yantanabie	96. Butler	154. Booyoolie	214. Moorooroo	269. Freeling
34. Finlayson	97. Dixon	155. Caltowie	215. Jellicoe	
35. Tarlton	98. Ulipa	156. Yangya		<i>Pinnaroo</i>
36. Cungenia	99. Cummins	157. Belalie	<i>Yorke</i>	270. Bowhill
37. Kaldoonera	100. Stokes	158. Whyte	216. Tickera	271. Bandon
38. Scott	101. Yaranyacka	159. Terowie	217. Wiltunga	272. Chesson
39. Murray	102. Warrow	160. Wandearah	218. Wallaroo	273. Mindarie
40. Chandada	103. Mortlock	161. Crystal Brook	219. Kadina	274. Allen
41. Karcultaby	104. Koppio	162. Narridy	220. Ninnies	275. Kekwick
42. Ripon	105. Huthison	163. Bundaleer	221. Kulpara	276. McGorrery
43. Forrest	106. Louth	164. Reynolds	222. Tiparra	277. Vincent
44. Campbell		165. Anne	223. Clinton	278. Wilson
45. Inkster	<i>Flinders</i>	166. Hallett	224. Kilkerran	279. McPherson
46. Wrenfordsley	107. Woolyana	167. Munderoor	225. Maitland	280. Peebinga
47. Rounsevell	108. Wonoka	168. Red Hill	226. Cunningham	281. Hooper
48. Witera	109. Arkaba	169. Koolunga	227. Wauraltee	282. Marmon Jabuk
	110. Warcowie	170. Yackamoor-	228. Muloowurtie	283. Molineux
<i>Eyre</i>	111. Adams	undie	229. Koolyurtie	284. Cotton
49. Wright	112. Wyacca	171. Andrews	230. Curramulka	285. Bews
50. Condada	113. Kanyaka	172. Ayers	231. Minlacowie	286. Parilla
51. Carina	114. Wirreanda	173. Kingston	232. Ramsay	287. Pinnaroo
52. Minnipa	115. Yarra	174. Wokurna	233. Para Wurle	288. Sherlock
53. Yaninee	116. Boolcunda	175. Barunga	234. Moorovyie	289. Roby
54. Pygery	117. Uroonda	176. Boucaut	235. Dalrymple	290. Peake
55. Wudinna	118. Eurilpa	177. Hart	236. Melville	291. Price
56. Travers	119. Pichi Richi	178. Milne	<i>Pyap</i>	292. Allenby
57. Wallis	120. Palmer	179. Hanson	237. Beatty	293. Livingston
58. Palabie	121. Mookra	180. Koorunga	238. Cadell	
59. Wannamana	122. Yanyarrie	181. Baldina	239. Markaranka	<i>Tatiara</i>
60. Mamblin	123. Bendleby	182. Cameron	240. Pooginook	294. Stirling
61. Kappakoola	124. Woolundunga	183. Everard	241. Parcoola	295. Wirrega
62. Warrambo	125. Willochra	184. Blyth	242. Murtho	296. Tatiara
63. Cortlinye				

* Names of regions are printed in italics.



OPENED PRE - 1896

TREND OF TYPE I OR II, FIGURE 10.....

TREND OF TYPE III, FIGURE 10.....

TREND OF TYPE IV OR V, FIGURE 10.....

TREND OF TYPE VI, FIGURE 10.....

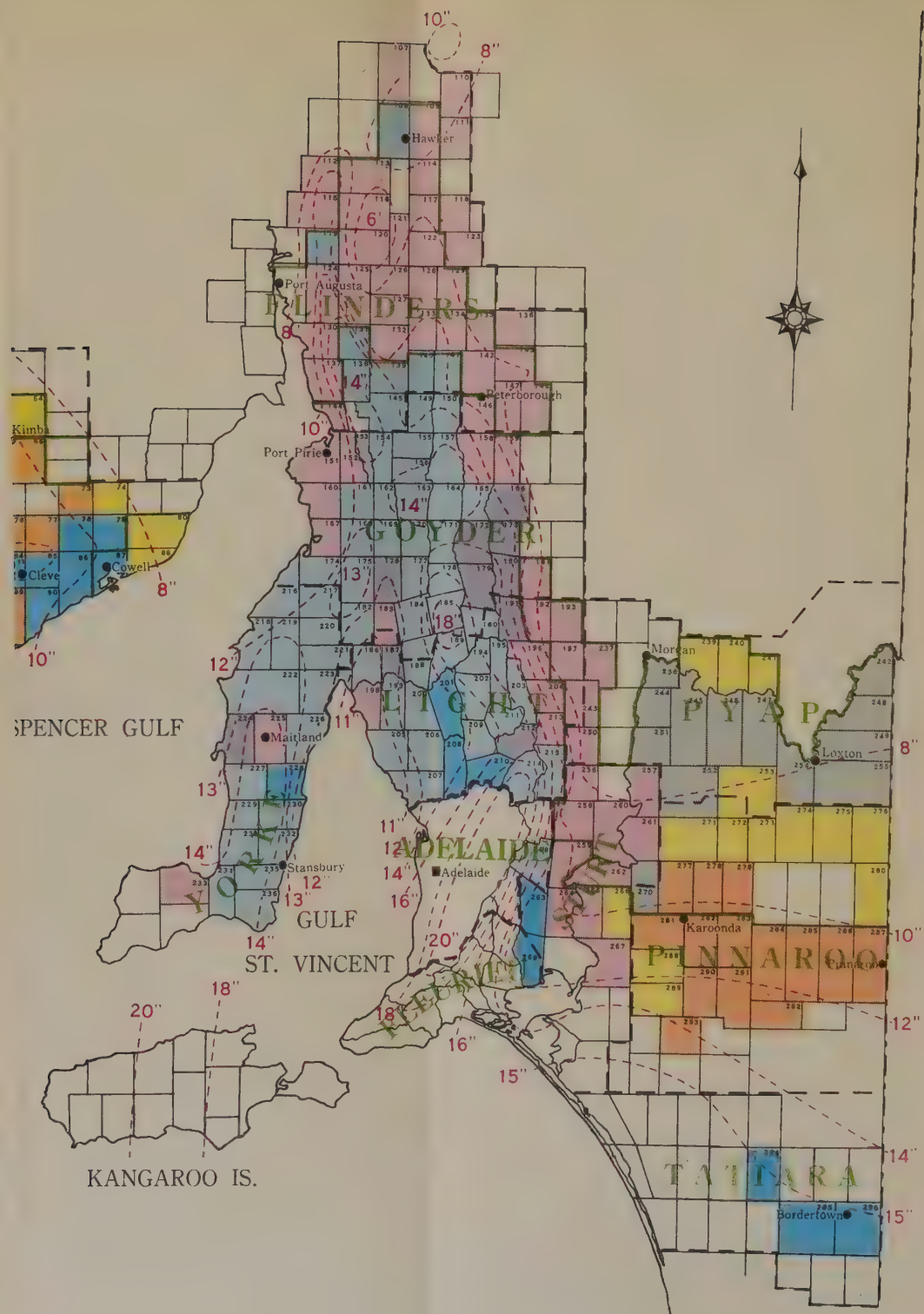
OPENED IN 1896 OR LATER

TREND OF TYPE I OR II, FIGURE 11.....

TREND OF TYPE III, FIGURE 11.....

TREND OF TYPE IV FIGURE 11.....

Fig. 12. Distribution of Yield Trends in



the Wheat Belt of South Australia.

In surveying the whole wheat belt, reference to both maps is greatly facilitated by using a *primary* subdivision into the somewhat artificial* regions of the Regional Planning Committee (loc. cit.), rather than a natural one based on soil type and rainfall. The year or period during which settlement took place is listed with each group of hundreds.

Nuys

1. Grey-brown loamy soils with affinities to the desert loams; rainfall approximately 10 in. throughout the group.

Generally speaking, the soils improve along the line from Fowler's Bay to a point just east of Penong.

a. Miller (1)†, Wookata (2), Sturdee (3), and Caldwell (4) (1896).—Although these hundreds were opened in 1896, analysis of the data could only be made from 1907, since all records prior to this date were included as one in the returns. During the first eleven years, yields may have increased slightly, but it is much more likely that they were only maintained. The curves show a steady decline from 1907, which is not typical of the group, possibly owing to a higher level of salinity in the soils and the fact that the areas under cultivation are too small to be representative. For convenience in mapping, they have been included

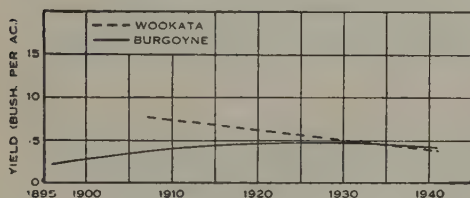


Fig. 13

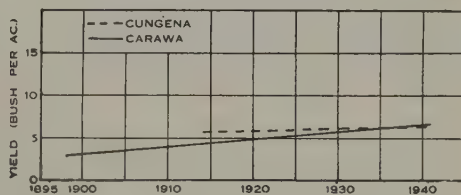


Fig. 14

as I(11). Figure 13 illustrates the smoothed trend in Wookata after variations due to seasonal rainfall have been eliminated, or as may be alternatively viewed, the course that yield would have followed if the rainfall variates had remained constant at their average values, throughout the period under examination.

b. Nash (5), Magarey (6), Giles (7), Cohen (8), Burgoyne (9), Bagster (10), Kevin (11), Keith (12), Catt (13), Horn (14), and Bartlett (15) (1890-1900).—For some 15-20 years yields slowly increased, but under the severe conditions this effect was transitory, and they subsequently declined, following courses I and II(11), according to local conditions. The curve for Burgoyne is also given in Figure 13. The influence of the slight improvement in the class of soil is shown by the sequence Wookata, Nash (the type I curve of Fig. 11), and Burgoyne.

2. Calcareous aeolianite; rainfall 9-10 in.

a. O'Loughlin (16), Moule (17), Goode (18), Bonython (19), Wandana (20), Chillundie (21), Guthrie (22), Blacker (23), Wallanippie (24), Carawa

* i.e. Artificial for present purposes.

† The numbers in brackets refer to the numbered hundreds on the maps (Figs. 8 and 12).

(26), Haslam (30), Perlubie (31), Finlayson (34), Ripon (42), Tarlton (35), Scott (38), and Murray (39) (1896).—The last three hundreds lie in the transitional zone between the calcareous sands and the adjoining stony mallee region; their trends resemble the remainder of the group, and consequently they have been included here. All yields show the expected increase, but as with the previous group, the effect is temporary, and they subsequently decline, the types being I and II(11) according to local conditions. The type II curve of Figure 11 is actually that of Perlubie. Carawa, with its type IV(11) curve, stands out as an exception. In this hundred, seasonal conditions are much better than indicated by the 10 in. isohyet because in the small section to which cropping has been restricted the rainfall is, on the average, nearly 11½ in. The maximum yield, attained in 1941, was, however, only about 7 bushels per acre.

b. Petina (27), Hague (25), Walpuppie (32), Cungenia (36), and Wallala (28) (1907-17).—In these hundreds the records are too short to make a definite decision regarding trend, but the yields show small increases, and this observation agrees with the view that the subgroup is following, some 10-20 years later, the first phase of the course set by the older districts on this class of soil. The trends have been classified as type III(11).

Hague, which was opened in 1911, declines from the outset; only a small area is cultivated, and it has probably been exploited fully. In Figure 12 this hundred has been included under type I(11). Figure 14 shows the courses of yield in Carawa and Cungenia.

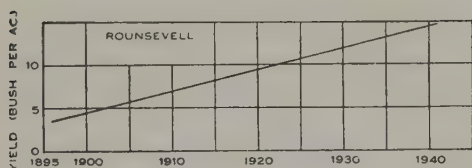


Fig. 15

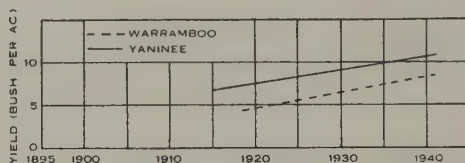


Fig. 16

3. Stony mallee; rainfall 10-13 in.

This group is mapped as stony mallee, but practically all the wheat is grown on scattered areas of sandy mallee which was derived from granitic parent rock.

a. Forrest (43), Wrenfordsley (46), Rounsevell (47), Witera (48), Campbell (44), and Wright (49) (1889-96).—With such a short period of light cropping prior to the advent of superphosphate, fertility was barely affected in these hundreds, and consequently no marked reaction occurs immediately after 1896. Yields steadily increase up to 1941, and all have been classified as type IV(11). These observations, coupled with the higher mean yields, provide a striking contrast with those made previously, and are directly ascribable to the more fertile class of soil and the additional 2-3 in. of seasonal rainfall. Figure 15 shows the course of yield in Rounsevell.

b. Travers (56) and Wallis (57) (from Eyre), Chandada (40), and Inkster (45) (1910-17).—Trends have not been definitely established, but the results

obtained agree generally with the remainder of the group. They have been classified as type III(11).

Eyre

1. Sandy mallee; rainfall 9-11 in.

All hundreds were opened for settlement during 1914-19, and are divisible into two subgroups, depending on the nature of the soils.

a. Koolgera (29), Yantanabie (33), Kaldoonera (37), Karcultaby (41) (from Nuyts), Condada (50), Carina (51), Palabie (58), Wannamana (59), Mamblin (60), Kappakoola (61), and Warramboos (62).—The trends are in accordance with the expectation that yields will improve for some years, but owing to the short cropping history only three have been definitely established, namely, Wannamana, Kappakoola, and Warramboos. It will be noted that the latter two hundreds lie in close proximity to the 11 in. isohyet. This trio has been classified as type IV(11), and the remainder as type III(11).

b. Minnipa (52), Yaninee (53), Pygery (54), and Wudinna (55).—The distinguishing feature of the soils in these hundreds is their granitic origin, and the greater fertility is reflected immediately in the higher mean yields, which are similar to those of Rounsevell etc. The trends of Yaninee and Pygery have been established as type IV(11) but those of Minnipa and Wudinna are not so marked, and they have accordingly been grouped as type III(11). Yaninee and Warramboos are illustrated in Figure 16, which shows clearly the 2-3 bushel advantage in yield conferred by the better soil.

2. Transitional mallee-solonetz soils; rainfall 10-13 in.

a. Barwell (69), McLachlan (70), Tooligie (82), Palkagee (71), Rudall (83), Roberts (89), Dixon (97), Smeaton (75), Pascoe (72), Campoona (76), Solomon (65), and Kelly (66) (1907-21).—The last two hundreds contain, in addition, areas of mixed mallee soils. Even though the records are comparatively short, it has been possible to establish the reality of the improvement in yield, since the soils are more fertile and the seasonal conditions more favourable. The whole set is outstanding and has been classified as type IV(11). Tooligie (0.30 bushel per acre per annum) and Palkagee (0.42 bushel per acre per annum) have remarkably high increments.

b. Boothby (90), and Yadnarie (84) (1880-84).—The light and spasmodic cropping in these hundreds during the first twenty years did not materially reduce fertility, and consequently there is no marked reaction following the first applications of superphosphate. Yields increase steadily up to 1941, following the course of type VI(10). Figure 17 contrasts the curves of Tooligie and Boothby; the superiority of the former with respect to mean yield and trend is largely due to the advantages conferred by a difference of approximately 2 in. in the rainfall.

3. Loamy mallee soils merging into red brown earths.

The occurrences of these soils are in two separate localities within zones of different rainfall.

(i) Rainfall 9-12 in.

a. Miltalie (78), Minbrie (79), Mann (85), Hawker (86), and Playford (87) (1880-84).—The remarks here are similar to those given for Boothby and Yadnarie, all curves being of type VI(10). The result obtained for Playford is surprising, but it is readily understood when account is taken of the changes in acreage. The greatest area recorded was 8600 acres in 1913, and since then it has progressively decreased, at the same time concentrating in the north-west

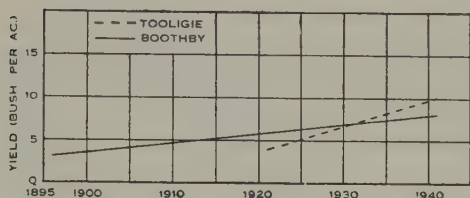


Fig. 17

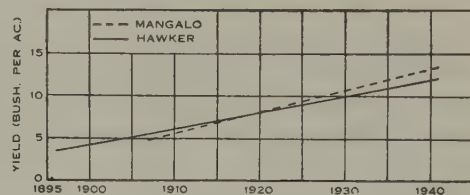


Fig. 18

corner of the hundred on loamy mallee soils. Associated with this progressive retreat to the better areas is a gradual increase in mean yield, the effect of which is to reverse the curvature of the regression on time, the net result being that yield exhibits a small but real improvement, but the maximum yield was only about 6 bushels per acre.

b. Mangalo (77) (1907).—The remarks here are similar to those given for Barwell etc., and the curve is of type IV(11).

Mangalo and Hawker, which are illustrated in Figure 18, resemble Tooligie and Boothby respectively. The loamy mallee soil is definitely superior to the transitional phase, but rainfall is the limiting factor.

(ii) Rainfall 12-16 in.

a. Stokes (100), Yaranyacka (101), Hutchison (105), and Louth (106) (1880).—From 1880 to 1896 only limited areas were sown to wheat, and to some extent cropping was also spasmodic. Very little deterioration occurred, so that the initial response to superphosphate is not strongly marked. All yields increase linearly, following curves of type VI(10).

b. Shannon (93), Brooker (94), Moody (95), and Butler (96) (1897-1907).—These hundreds all possess well-defined courses of type IV(11), Shannon, in particular, having the high annual increment of 0.40 bushel per acre. The curves of Hutchison and Shannon are given in Figure 19. Comparison of Figures 18 and 19 brings out clearly the superiority of the group in the zone of higher rainfall, where all rates of increase are greater both relatively and absolutely. This small section constitutes one of the most progressive portions of the wheat belt.

4. Pod sols; rainfall 16-20 in.

a. Warrow (102), Ulipa (98), and Koppio (104) (1880).—The three curves are of type VI(10).

b. Cummins (99) and Mortlock (103) (1907).—These hundreds have been classed as type IV(11).

The series possesses an interesting feature, of which no trace is present in any other district. Neither subgroup shows an improvement in yield at the beginning of the several periods examined; in the three hundreds that were settled first, yield was maintained at 6-8 bushels per acre and did not increase

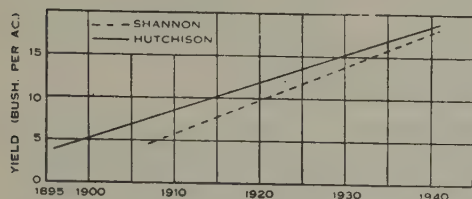


Fig. 19

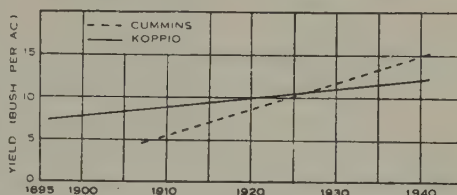


Fig. 20

until some years after the introduction of superphosphate, and in the remaining two, the improvement likewise did not follow until after an initial stationary period. For the purposes of classifying and mapping the trends, this small departure from linearity was ignored. Figure 20 illustrates the courses in Koppio and Cummins.

5. Rendzinas; rainfall 12-16 in.

a. Colton (67), Talia (68), Ward (81), and Kiana (91) (1880).—As with other districts on Eyre Peninsula, in which early settlement occurred, initial cropping made only light demands on the soil, so that no marked changes occurred in these hundreds shortly after the first applications of superphosphate. From 1896, all yields increase steadily, following curves of type VI(10).

b. Mitchell (92) (1907).—In this area the curve is of type IV(11).

The group, as a whole, has a higher rainfall than the loamy mallee soils of the Cleve district, but the annual increments in yield, although quite significant, are only of the same order as those of the latter, i.e. about 0.10 bushel per acre per annum.

6. Loamy mallee soils agriculturally similar to the desert loams; rainfall 8-10 in.

Wilton (88), Glynn (74), Warren (80), Moseley (64), and Cortlinye (63) (1907-16).—This series has been classed as type III(11), since a definite decision regarding trend has not been obtained. Moseley and Cortlinye are the only important areas in the group.

7. Solonetz; rainfall 10 in.

The only hundred concerned is James (73), which was opened for settlement in 1910. The increase in yield is small (about 0.10 bushel per acre per annum) but definite, and has been included under type IV(11).

Flinders

1. Desert loams and mixed mallee types merging into desert loams; rainfall 6-10 in.

This group includes all hundreds in Flinders that are not listed under sections 2, 3, and 4 below (1875-84).

2. Sandy mallee; rainfall 10-12 in.

Winninowie (130), Baroota (137), and Telowie (144) (1875-84).

3. Red brown earth and mixed mallee; rainfall 10-12 in.

Black Rock Plain (141), and Yongala (146) (1875-84).

In the three groups, exploitative management of the first twenty years rapidly depleted soil reserves, yields declined, and incipient erosion developed. With the beginning of the new century came a full realization that in addition to the inferior types of soil, the settler had to contend with an extremely erratic seasonal rainfall. In consequence, the boundary of the area under cultivation was withdrawn during 1902-09, and cropping confined to scattered localities in the Flinders Ranges, which possessed arable pockets of somewhat better soils with a slightly higher rainfall. Superphosphate and the retreat to the more favourable sites were the principal factors responsible for the increase in yield

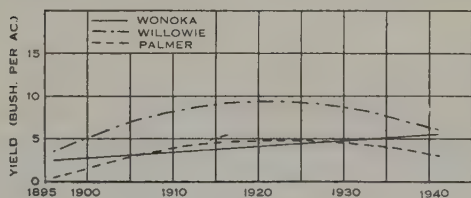


Fig. 21

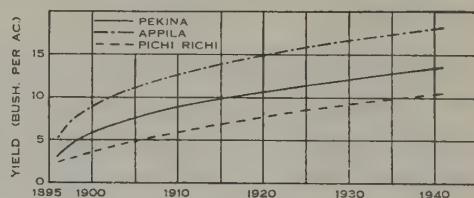


Fig. 22

from 1896 to 1920, but this effect was transitory, since, with very few exceptions, even in these situations, the soils were incapable of withstanding frequent cropping. From about 1920, yields again declined, and apart from Wonoka, type V(10), and Black Rock Plain, type IV(10), the courses are of types I and II(10), depending upon local conditions. Wonoka and Black Rock Plain supply good illustrations of the effect on the trend in yield of a progressive reduction in acreage and concentration on better soils; in the latter almost the entire area occupied by the crop is red brown earth. In the majority of hundreds with type II curves the area of wheat was small and remained constant, the inference being that cultivation had been restricted to slightly better situations throughout. Palmer, Willowie, and Wonoka are contrasted in Figure 21; the type II curve of Figure 10 is that of Yongala.

4. Red brown earth; rainfall 10 in. to more than 14 in.

Pichi Richi (119), Gregory (131), Wongyarra (138), Booleroo (139), Appila (145), and Pekina (140) (1875).—After settlement, the area in each hundred increased to a point where it became stable. The initial exploitative

cropping caused yields to decline, but they recovered after the introduction of superphosphate, and under the greatly improved conditions of soil and rainfall, continued to increase up to 1941, although at diminishing rates. Pichi Richi and Wongyarra are of type V(10), and the remainder type IV(10). In Pichi Richi the area of crop has remained practically constant since 1880, and actually only part of the hundred is classed as marginal land, but for convenience in mapping this has not been indicated in Figure 12. Figure 22 shows the courses in Pichi Richi, Pekina, and Appila, and illustrates the effect of an increase in seasonal rainfall from 10 in. to 14 in. The type V curve of Figure 10 is that of Wongyarra.

Goyder

1. Mixed mallee; rainfall 9-12 in.

Terowie (159), and Baldina (181) (1875-84).—The history of cropping in these hundreds runs exactly parallel with that of the hundreds with type I curves in Flinders; Baldina is type I(10), and Terowie type II(10), the latter being almost identical with the curve for Yongala in Figure 10.

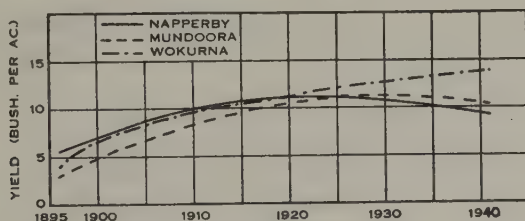


Fig. 23

2. Sandy mallee; rainfall 10-13 in.

Pirie (151), Wandearah (160), Napperby (152), Mundoorra (167), and Wokurna (174) (1875-84).—In all districts the yields show the characteristic decline prior to 1896, and subsequent recovery which is transitory except in Wokurna. Rainfall increases from 10 in. to 13 in. in the order in which the hundreds are named, and the trends follow, in the sequence of types I, I, II, II, and IV(10), with improvement in the soils. The course in Pirie is the type I curve of Figure 10, Wandearah is closely similar, with a maximum of 11 bushels per acre, and Napperby, Mundoorra, and Wokurna are given in Figure 23. Winninowie, Baroota, and Telowie (from Flinders) also fall properly into the sequence, their curves resembling that of Napperby.

3. Loamy mallee; rainfall 11-16 in.

Crystal Brook (161), Narridy (162), Yangya (156), Koolunga (169), Boucaut (176), Cameron (182), Everard (183), and Blyth (184) (1870-84).—All yields exhibit marked deterioration prior to 1896 and the subsequent recovery, but the curves vary according to the local conditions. Everard is of type I(10), and Boucaut type III(10). The soils of Everard are variable, and often poorer than the average grade of loamy mallee, while in Boucaut a small tract of inferior

stony mallee occurs; in addition, both hundreds are endoreic zones in which problems, associated with the development of salinity in the surface soils, have arisen. The influence of the more fertile class of soil is apparent in the remaining hundreds, all of which are of type IV(10). Figure 24 shows the courses in

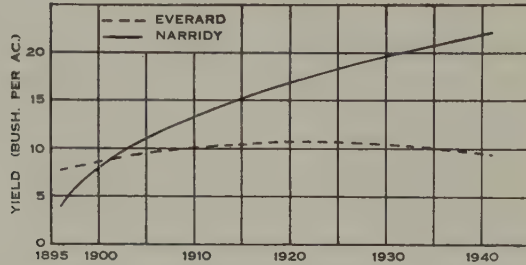


Fig. 24

Everard and Narridy, and the type III curve of Figure 10 is that of Boucaut.

4. Red brown earth; rainfall 12-18 in.

Tarcowie (149), and Mannanarie (150) (1870-75); Howe (153), Booyoolie (154), Caltowie (155), Belalie (157), Whyte (158), Bundaleer (163), Reynolds

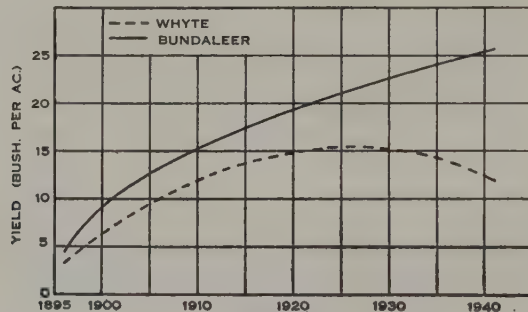


Fig. 25

(164), Anne (165), Red Hill (168), Barunga (175), Yackamoorundie (170), Hart (177), Ayers (172), and Andrews (171) (1866-75); Milne (178) and Hanson (179) (1860-70); Kingston (173), Koorunga (180), and Hallett (166) (1875-84); and Clare (185) (1860).—All trends display remarkably high rates of increase for some ten years after the initial applications of superphosphate, the general curve being type IV(10), but there are several exceptions. Whyte, type II(10), is comprised partly of mixed mallee soils, and just over half the area under wheat lies in a zone between the 10 in. and 12 in. isohyets. The acreage has been maintained throughout and this is the main factor underlying the contrast with Black Rock Plain. Ayers, Hanson, Hallett, Kingston, and Koorunga follow curves of type III(10). The best soils of these five hundreds are devoted principally to lucerne, and wheat is relegated to the lower slopes of the hills on shallow residual red brown earths, which are less fertile than the

alluvial types of Belalie and Caltowie, for example. Hallett, Kingston, and Kooringa are extreme in this respect, while Ayers and Hanson carry an inter-grade soil between the extremes. Whyte and Bundaleer are contrasted in Figure 25. Clare follows a course of type V(10).

Light

1. Mixed mallee

(i) Rainfall 6-10 in.

Bright (192), and Bunday (193) (1880); and Bower (197) (1884).—This trio forms part of the worst section of the wheat belt. The curves are type I(10), and the history of cropping is similar to that of hundreds in Flinders with the same form of trend.

(ii) Rainfall 10-14 in.

English (196), Neales (204), and Dutton (213) (1860-70).—In these cases the mean yields are considerably greater, and the curves are type II(10). Bower, Neales, and English are compared in Figure 26, which shows clearly the effect of the additional 4 in. seasonal rainfall. The course of yield in Dutton is almost identical with that of English.

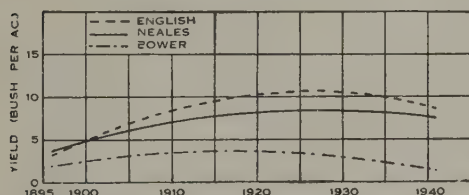


Fig. 26

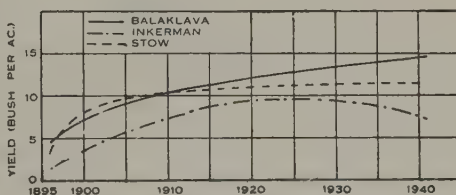


Fig. 27

2. Sandy mallee; rainfall 10-14 in.

Inkerman (198), Balaklava (199), Hall (188), Goyder (186), and Stow (187) (1860-70).—In Inkerman, where conditions are similar to those of Mundoorra, the curve is of type II(10) and falls properly into sequence with other members of the sandy mallee group immediately to the north. Problems associated with the development of salinity in the surface soils similar to those in Boucaut, also occur in Stow, and the resulting curve is of type III(10). The remaining hundreds are of type IV(10). Figure 27 gives the trends of Inkerman, Stow, and Balaklava.

3. Loamy mallee; rainfall 11-14 in.

Port Gawler (207), Dublin (205), Grace (206), Dalkey (200), and Mudlawirra (208) (1850-70).—The first four hundreds provide, in the order given, a sequence of improving variants of type IV(10), and Mudlawirra is type VI(10). In the latter, wheat was the dominant crop during the early years, reaching its peak in the decade 1870-80, when nearly 30,000 acres were cropped. The area was reduced to 10,000 acres by 1896, and since then has varied about this value. The general tendency has been to pass from wheat as

the major enterprise to mixed farming, with an increase in the number of stock carried, accompanied by an increase in the length of rotations.

4. Red brown earth; rainfall 14-18 in.

Upper Wakefield (189), Stanley (190), Alma (201), Saddleworth (194), Gilbert (202), Waterloo (195), Julia Creek (203), Kapunda (211), Light (209), Belvidere (212), Nuriootpa (210), Moorooroo (214), and Jellicoe (215) (1850-66); and Apoinga (191) (1866-70).—Apoinga and Belvidere are of type III(10). The former is a member of the series Hallett etc., for the same reasons as given previously, while in the latter, vineyards occupy the best soils and wheat is cropped on intermediate grades of red brown earth resembling those of Ayers and Hanson. Alma and Nuriootpa follow courses of type VI(10), Moorooroo is of type V(10), and the remainder type IV(10). The position with

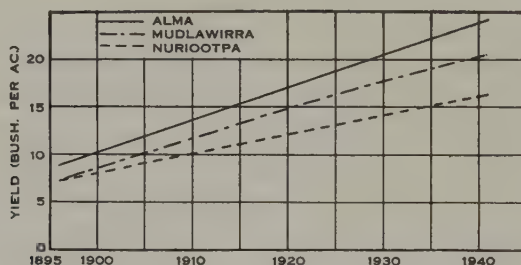


Fig. 28

respect to wheat as the major crop has undergone a radical change in Nuriootpa and Alma, more particularly in the former. In both hundreds the area decreased from its maximum, about 25,000 acres, attained during 1870-80, and by 1896 had fallen to 10,000 acres. With the appearance of superphosphate, the area increased to 15,000 acres in Alma, and remained approximately constant up to 1941. This hundred possesses particularly good soils, and with the increase in the number of stock carried there has been a trend to mixed farming under longer crop rotations. On the other hand, in Nuriootpa the area of crop was reduced still further, until it roughly stabilized at 5000 acres. During the period under review, the area devoted to vineyards has increased, and wheat has been replaced as the major crop, practically all of it having been produced under mixed-farming conditions.

Mudlawirra, Alma, and Nuriootpa are illustrated in Figure 28.

Yorke

1. Sandy mallee; rainfall 12-13 in.

Wiltunga (217), and Ninnes (220) (1875-84).—These two hundreds are situated at the southern extremity of the sandy mallee zone running parallel with the eastern coastline of Gulf St. Vincent. The curves are type IV(10) and follow in sequence after Wokurna in conformity with the higher rainfall and improvement in the soils.

2. Loamy mallee; rainfall 12-16 in.

Maitland (225) (1870-75); and Tickera (216), Wallaroo (218), Kadina (219), Tiparra (222), Kulpara (221), Clinton (223), Kilkerran (224), Wauraltee (227), Cunningham (226), Muloowurtie (228), Koolywurtie (229), and Curramulka (230) (1875-84).—In Kilkerran, salinity problems have developed as in Boucaut and Stow, and the yield follows a curve of type III(10). Muloowurtie is type VI(10), and although first settled during 1875-84, extensive development has only occurred in recent years, the effect of a progressive introduction of virgin land being to reverse the curvature of the regression on time, and thus maintain the rate of increase in yield. The results for Maitland, type II(10), with a maximum in 1938, and Curramulka, type V(10), in which the trend had nearly reached zero in 1941, are of far-reaching importance, and are directly ascribable to intensive cropping of cereals in the area. The remaining hundreds are type IV(10). Maitland, Tiparra, Curramulka, and Muloowurtie are illustrated in Figures 29 and 30.

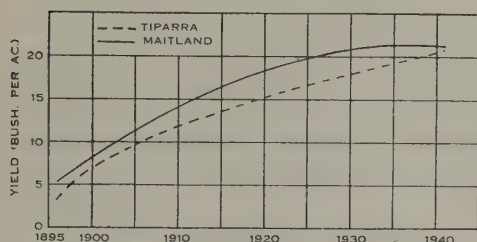


Fig. 29

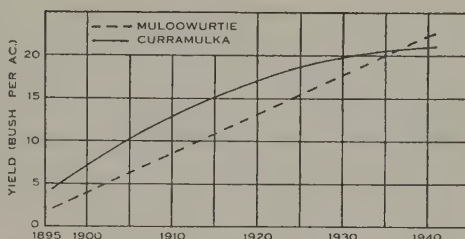


Fig. 30

3. Rendzina; rainfall 14-16 in.

Minlacowie (231), Dalrymple (235), Melville (236), Moorowie (234), Para Wurlie (233), and Ramsay (232) (with some sandy mallee) (1870-80).—Moorowie and Ramsay are type IV(10), Para Wurlie is type II(10), and the remainder type V(10). The curve for Para Wurlie is an extreme variant of type II(10) in which the trend reached zero in 1940. Trace-element deficiencies are known to occur in this locality (*vide infra*).

As in southern Flinders and Goyder, the yields of all hundreds in Light and Yorke declined markedly during the period of cropping prior to 1896, and in general showed very high rates of recovery for some years after the initial applications of superphosphate. The group of four regions constitutes that part of the wheat belt which suffered most severely under the stringent conditions of cropping prior to 1896.

Sturt

1. Red brown earth; rainfall 12-14 in.

Monarto (263) and Tungkillio (268) (from Adelaide) (1860); and Freeling (269) (from Fleurieu) (1862).—In these hundreds there are occurrences of mixed mallee soils, but the bulk of the wheat is grown on red brown earth, and through-

out the long history of cropping none of them has carried more than 3-5 thousand acres of wheat. The types are Monarto and Freeling VI(10), and Tungkillo V(10). Figure 31 illustrates Monarto.

2. Sandy mallee; rainfall 9 in.

Forster (261), the only hundred concerned, was opened in 1884. Yields show the characteristic decline prior to 1896, and the subsequent temporary recovery. The course of yield is type II(10), closely similar to the curve for Gordon in Figure 34.

3. Stony mallee; rainfall 8-12 in.

a. Ridley (260), Nildottie (257), Younghusband (262), Burdett (265), and Seymour (267) (1880).—The remarks here are similar to those made for Forster, all curves being of type II(10).

b. Ettrick (266) (1910).—The trend of yield during the shorter period of cropping in this hundred resembles the first phase of the remainder on this soil

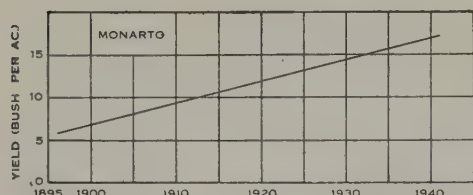


Fig. 31

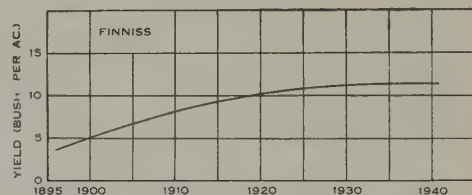


Fig. 32

group, and has accordingly been classified as type III(11).

4. Mixed mallee; rainfall 8-12 in.

Bagot (256), Angas (258), Finnis (259), and Mobilong (264) (1862-75).—The courses of yield, which are of type II(10), are similar to those of English, Neales, and Dutton, on the same class of soil. Finnis is illustrated in Figure 32, and the curve for Mobilong is almost identical.

Pyap

1. Stony mallee; rainfall 6-10 in.

a. Anna (250), Brownlow (243), and Beatty (237) (1870-84).—These hundreds, together with Bagot from Sturt, Baldina from Goyder, and Bright, Bunday, and Bower from Light, comprise the worst section of the wheat belt. The three type I(10) curves are similar to that of Bower in Figure 26.

b. Cadell (238), Murbko (244), Paisley (251), and Bakara (252) (1896); and Mantung (253) (1909).—Yields of the first four hundreds show the expected increase, but as with all other mallee soils under similar conditions of rainfall, this effect is temporary. The curves are all of type II(11). Mantung, with its shorter cropping history, has improved slowly, following a course similar to the first phase of the curves in the older districts. The trend is not significant and has been classed as type III(11). The curves of Cadell and Mantung are given in Figure 33.

2. Sandy mallee; rainfall 6-8 in.

Waikerie (245) (partly stony), Holder (246), Moorook (247), Gordon (249), Pyap (254), and Bookpurnong (255) (1896); and Markaranka (239), Parcoola (241), and Pooginook (240) (1913).—The remarks here are similar to those of the previous section. Waikerie is type I(11), the next five are type II(11), and the last three type III(11). The curve for Gordon is given in Figure 34.

3. Mixed mallee; rainfall 6-8 in.

Murtho (242) and Paringa (248) (1896).—The curves are type I(11) and remarks similar to those above.

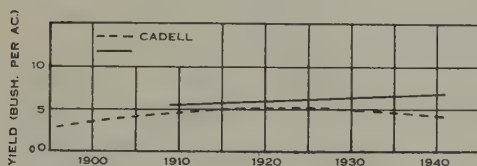


Fig. 33

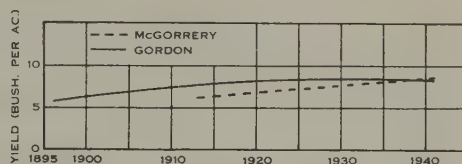


Fig. 34

Throughout the region considerable reductions have been made in acreage since 1930, the nett effect of which has been to reduce the rate of decline in yield.

Pinnaroo

1. Sandy mallee; rainfall 8-10 in.

Bowhill (270) (with some stony and transitional mallee) (1896); and Bandon (271), Chesson (272), Mindarie (273), Allen (274), Kekwick (275), and McGorrrery (276) (1907-19).—The curve for Bowhill is type II(11); and the remainder are of type III(11); remarks are similar to those above. McGorrrery is shown in Figure 34.

2. Transitional mallee-solonetz; rainfall 10-12 in.

Vincent (277), Wilson (278), McPherson (279), Hooper (281), Marmon Jabuk (282), Molineux (283), Sherlock (288), Roby (289), Peake (290), Price (291), Cotton (284), Bews (285), Parilla (286), Pinnaroo (287), Peebinga (280), and Allenby (292) (1907-19).—The general curve is type IV(11) with the exception of Peebinga, Roby, and Sherlock, which are type III(11). This soil group is outstanding, and the contrast between it and neighbouring mallee types to the north is just as strongly marked as on Eyre Peninsula. The courses of yield in Pinnaroo and Roby are illustrated in Figure 35.

3. Solonetz; rainfall 14 in.

The only hundred concerned is Livingston (293), which is also of type IV(11) and was opened in 1907.

Tatiara

1. Solonetz; rainfall 15 in.

Stirling (294) (1889).—The curve is type VI(10), and the marked superiority of this hundred over Livingston is due to the fact that wheat is confined to a

section in which the solonetzic soils are mixed with a rendzina-terra rossa complex of greater fertility.

2. Wimmera grey loam; rainfall 15 in.

Tatiara (296) and Wirrega (295) (1876 and 1877).—In both hundreds yields declined slightly under the cropping prior to 1896, but after the introduc-

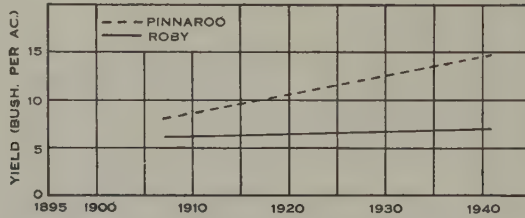


Fig. 35

tion of superphosphate increased steadily up to 1941, following curves of type VI(10). Stirling and Wirrega are contrasted in Figure 36, which shows the superiority of the Wimmera loam; the difference is even more marked when a comparison is made between Tatiara, the type VI curve of Figure 10, and Stirling.

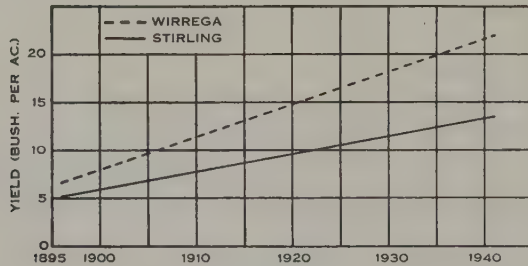


Fig. 36

The remarkable progress made in these districts agrees with observations made by Wadham and Wood (1939) on the Victorian counterpart of the soils.

(d) Discussion of Trends

It must be emphasized that this study is, by nature, only a broad survey. Obviously, with respect to any particular hundred there is, on the one hand, a set of factors tending to increase yields, and on the other, a set tending to decrease them. The observed trend is the resultant of their effects, and because of the nature of the data they are completely confounded and incapable of separate assessment; in fact, it is not even possible to enumerate either set exhaustively. But in viewing the matter broadly, some indication can be given of the major contributors in the two directions. Several instances have already been given in Section V(c).

On the positive side the principal factors are:

1. Maintenance of an adequate phosphorus and nitrogen supply.
2. Adoption of cultural practices suited to various types of soil.
3. Use of improved varieties adapted to the particular conditions of different localities.
4. Maintenance of the physical condition of the soil.

On the other hand, decreases can be unhesitatingly ascribed to a reduction of fertility consequent upon agricultural exploitation of the soils through over-cropping in short-term rotations. As pointed out above, this has proceeded so far in some areas that it outweighs the beneficial effects of recent advances, and results in a progressive decline. In other localities, although yield was still increasing in 1941, it would appear that farming practices in vogue were none the less exploitative, since rates of increase were being rapidly decelerated and yields were not as high as would be anticipated considering the nature of the soils, the seasonal rainfall, and the potentialities of the latest varieties at the time. Further reference is made to this point below.

The decline in fertility is attributable to (i) a cumulative deficiency of one or more essential elements, and (ii) a loss of soil structure. Each of these may exert its effects directly or indirectly.

(i) *Cumulative Deficiencies*.—Considering the soils of the wheat belt generally, no extensive quantitative evidence has hitherto been presented to show that there is a cumulative deficiency of any element which is essential in the assimilatory processes of the wheat plant, such as occurred with phosphorus. The paramount importance of nitrogen in this connection was obscured until about fifteen years ago by the fact that all experimental work had been conducted on fallowed land under conditions which were not conducive to substantial responses from nitrogenous fertilizers (Richardson and Gurney 1933). The necessity of fallowing for securing high returns received general recognition early in the development of the industry, and the practice was adopted widely. For many years its greatest advantage was considered to be conservation of water that fell in the fallow season, but latterly this view has changed. Observations which were assembled and reviewed by Prescott (1933) showed that nitrate accumulation is at least equally as important as moisture conservation and the extermination of weeds. It is known that the production of nitrate in a fallow is considerable (Prescott loc. cit.), and under optimal conditions is sufficient to meet the needs of heavy crops, i.e. 50-60 bushels per acre. Since, for example, a 15 bushel crop removes approximately 20 lb. nitrogen per acre in its grain and straw, and to this must be added the amounts lost in other ways, the maintenance of fertility, provided no other factor is limiting, rests upon the replacement of this nitrogen. Nitrogenous fertilizers are not used, so that unless natural sources exist to compensate the loss, it must be borne by the nitrogen reserves of the soil.

Three contributors that may be considered in this connection are:

1. Non-symbiotic nitrogen fixation.
2. Symbiotic fixation with legumes.
3. Nitrogen introduced in rain water.

With respect to the first, the weight of evidence at the present time points to the fact that it is not of any consequence in the soils of the wheat belt. Thus Beck (1935), after examining South Australian soils, concluded that Lewcock's (1925) claim of the universal distribution of *Azotobacter* was not substantiated, and even in soils containing the organism the numbers present were so small that the amounts of nitrogen fixed would be negligible. Swaby (1939) working with Victorian soils found *Azotobacter* comparatively rare, and reached the same conclusion as Beck. On the other hand, *Clostridium butyricum* was much more widely distributed, and present in greater numbers, but environmental conditions in the wheat soils are not suitable for the development of this anaerobe to the point where it could fix appreciable amounts of nitrogen. Finally, Jensen (1939), in reviewing current work at the time on New South Wales soils, concluded that non-symbiotic fixation is only "a minor and mostly insignificant factor in maintaining the nitrogen content of soils under wheat cultivation." Recent observations made by Clarke and Marshall (1947) are also relevant in this connection; and at the same time demonstrate forcibly the outstanding weakness of the fallow-wheat rotation. These authors determined the reduction in the nitrogen content (expressed as a percentage of air-dry soil passing a 2 mm. sieve) of two slightly different red brown earths, after various periods of cultivation. The total declines in surface soils (top 4 in.) were 0.158 to 0.094 per cent. and 0.222 to 0.135 per cent. after 16 and 20 years, respectively, of cropping in a fallow-wheat rotation with superphosphate applied to the crop. The upper limit of each range is for virgin soil, and both reductions, of which the greater part occurred in the first five years, are statistically significant. Declines in subsurface soils were not significant. No legumes developed on either site, and consequently any nitrogen which may have been added to the soils came from other sources. Such contributions, if they existed, must, however, have been very small, since the ratio of carbon to nitrogen in these soils is approximately 12, thus making the nitrogen content a fairly accurate index of their organic matter status. It would appear then that the nitrogen withdrawn by successive crops was derived principally from the organic reserves of the soils.

Symbiotic fixation of nitrogen with legumes has undoubtedly been of some importance in certain areas of the wheat belt; further reference to this point is relegated to a later stage of the discussion.

Jensen (loc. cit.) quotes amounts of 3-4 lb. nitrogen per acre per annum in rain water, but these are trivial.

The soils of the South Australian mallee have been studied by Prescott and Piper (1932), and the red brown earths by Piper (1938). The percentage of nitrogen in the surface soils of the mallee group ranges from low (less than 0.10 per cent.) to moderate (0.10 to 0.30 per cent.) in passing from light textured sandy types to the heavier loams, with an average of 0.06 per cent., but texture

and percentage nitrogen are not strongly associated; 87 per cent. of surface soils examined contained less than 0.10 per cent. nitrogen, and 83 per cent. of subsoils less than 0.05 per cent. In the red brown earths, the proportion of nitrogen varied from low to moderate, with an average of 0.10 per cent. for surface soils, 60 per cent. of which contained less than the average. The first 9 in. was taken in this group as surface soil, and this accounts for the fact that the average value lies at the lower extremity of the range for moderate amounts. The original reserves of nitrogen in the principal wheat soils are thus, at best, only moderate, and consequently it would not be surprising that an exploitative system of cropping has largely exhausted them.

Reference may be made to recent work on trace-element deficiencies, as this may also have a bearing on the nitrogen problem. The malady known as "coast disease" of sheep occurs in parts of South Australia, and has been shown to result from a dual deficiency of copper and cobalt in the fodder consumed by animals depastured in the affected areas (Marston, Lee, and McDonald, 1948*a*, 1948*b*). Other regions in which a deficiency of copper is not complicated by one of cobalt have been identified. On these much greater areas, the deficiency of copper varies in degree which is manifested by the animal in a range of symptoms, the first to appear being a lesion in the wool which is characteristic, specific, and easily discernible (Marston, Lee, and McDonald loc. cit.). The occurrence of these nutritional disorders in stock has led to intense work on the mineral requirements of oats and pasture plants, including legumes, in certain isolated areas (Riceman 1946, 1948), where essential trace elements, in particular copper and zinc, as well as phosphorus and nitrogen, are in extremely short supply. No widespread deficiencies have been observed with cereals, but observations made by Lee (personal communication, 1948) have shown that, except for the greater part of Flinders, copper deficiency, as indicated by the occurrence of lesions in wool, occurs throughout the portion of South Australia illustrated in Figures 8 and 12 and the extreme south-eastern section not included in the figures. The density of occurrence is greatest on the littoral calcareous dunes and adjacent areas, and is gradually reduced in passing from the mallee soils and genetically related types (Crocker 1946) to the red brown earths. As the requirements of the animal relative to those of the plant are enormous, it is much more sensitive and this would account for the observed facts. On the other hand, it is known that copper is essential in nitrification, and the possibility exists that in some areas after a period of cropping the originally low copper status of the soils has been reduced to a point where nitrifying organisms react to deficiencies which do not restrict the wheat plant. If this is so, it would tend to make nitrogen a limiting factor for wheat production; and of the main wheat soils, the mallee types are the most likely to be affected. Other elements are also required in nitrification, but particular reference is made only to copper in view of its known deficiencies.

There are also indications that deficiencies of certain elements are causing indirect effects by restricting symbiotic fixation of nitrogen. Trumble (personal communication, 1948), using various legumes as test plants, has obtained definite

responses in yield to the elements potassium, copper, zinc, molybdenum, boron, and manganese at widely dispersed points of the wheat belt, either singly (potassium, molybdenum, zinc) or in certain combinations.

The extent to which these factors operate in limiting the nitrogen supply can, however, only be resolved by an extensive set of field trials.

(ii) *Loss of Soil Structure*.—Frequent cropping leads also to deterioration of soil structure and, indirectly through this, to losses of plant nutrients by erosion. The reduction in water-stable aggregates originally present in virgin soil by cultivation has been well established, and it will suffice to refer to the work of Clarke and Marshall (*loc. cit.*). Changes in water-stable aggregation of red brown earth surface soils, resulting from increasing periods of cultivation up to twenty years, were measured, and significant decreases found in all cases. The major part of the decline took place in the first five years of cultivation, and the total declines ranged from 42 to 69 per cent. of the aggregates in virgin grassland. The decrease results directly from the mechanical action of tillage, and indirectly through the decomposition of the organic matrix during fallowing. The effect on soils of heavy texture is to reduce their absorptive and retentive powers in coping with heavy rains; surfaces tend to set hard, causing uneven germination and making cultivation more difficult. Piper (*loc. cit.*) records such instances in the zone of the red brown earths. The most serious consequence, however, is that water erosion supervenes and may advance to its worst form—sheet erosion. Considerable areas in Flinders, Goyder, and Light have been eroded in varying degree (Rural Reconstruction Commission 1944), and the position in the hundred of Belalie may be taken as typical of a large proportion of the best agricultural land:

Degree of Erosion	Per Cent. of Surface Soil Remaining	Per Cent. of Total Arable Land
Slight	> 75	24
Moderate	25-75	69
Severe	< 25	7

On light textured sandy soils, removal of the original vegetal cover followed by frequent cropping has led to sand drift by aeolian action, the principal regions concerned in this respect being Sturt, Pyap, and northern Pinnaroo.

(iii) *General Relation between Form of Trend and Nitrogen Status of the Soils*.—The orderly geographical distribution of the various types of trend is striking, and accords generally with the view that after the deficiency of phosphorus has been overcome the progressively diminishing nitrogen status of the soils is a dominant factor influencing the form of the curves.

On sandy, stony, and mixed mallee soils, and related types that were opened in 1896 or several years later, the onset of a decline appears after 20-25 years of cropping with adequate supplies of superphosphate, and by 1941 yield in the majority of hundreds concerned had fallen to approximately the value it had attained at the time of settlement. In these soils, the proportion of nitrogen in their virgin state is low and the losses sustained by exploitation during the pioneer-

ing years and later cropping, together with those due to erosion where it has occurred, are great enough to exhaust the reserves in approximately 45 years.

Similar mallee types, and the desert loams which were settled between 1875 and 1896, had been largely abandoned after 10-20 years, and consequently are not represented among the curves of Figure 10. After the advent of superphosphate, the remaining small isolated areas of slightly better soils carried on for a further 20 years, when a second decline supervened and yield fell gradually to its value at the beginning of the century (hundreds with curves of type I(10)). In these soils exhaustion thus occurs after 55-65 years of cropping.

TABLE 3
FREQUENCY DISTRIBUTION OF SEASONAL RAINFALL AT SELECTED STATIONS

Station	Length of Record (years)	Rainfall (in.)						Mean Apr.- Nov.
		> 14	12-14	10-12	8-10	6-8	< 6	
Fowler's Bay	69	6	8	15	25	12	3	10.22
Ceduna	39	1	4	7	10	13	4	8.89
Petina	38	4	8	12	11	1	2	10.68
Cortlinye	26	3	4	3	8	7	1	9.66
Cowell	65	2	6	9	18	21	9	8.51
Hawker	64	6	6	10	16	11	15	9.25
Hammond	59	2	6	4	14	15	18	8.14
Orroroo	73	8	11	15	15	17	7	10.00
Terowie	64	7	9	11	17	14	6	9.96
Sandleton	57		4	4	20	9	20	7.54
Morgan	54		3	2	9	16	24	6.67
Loxton	37	2		5	9	9	12	8.01
Copeville	34		5	7	8	7	7	8.82

All diagrams given above, which refer to these districts, indicate that the maximum yields obtained under average rainfall are extremely low; considerable effort would have to be expended to increase them by 50 per cent. Without doubt, deterioration could be arrested and fertility restored in certain localities, but considering the areas generally, improvement would be of limited extent, as the steps taken must ultimately depend upon rainfall and its reliability. At the moment, research is proceeding on this vitally important feature of the climate, but no detailed quantitative data are available for the areas concerned. The approximate analysis of records from typical stations, set out in Table 3, illustrates the extremely hazardous nature of the seasonal rains, and requires no further comment, except perhaps to add that all stations are presented in the most favourable light, since the average effective rainfall at each is only slightly greater than 5 in. (*vide* Trumble loc. cit., map 2).

As pointed out above, the large tracts of these particular mallee soils that have been developed recently appear to be following courses similar to the districts with longer records, and although yields were still slowly increasing at

the close of the period under review, it can be confidently anticipated that if cropping is continued under the old system, they will eventually decrease.

In an extensive and detailed report, the Pastoral and Marginal Agricultural Areas Inquiry Committee (*loc. cit.*) reviewed all enterprises within the marginal areas, and after examining the economics of wheat production both from the point of view of direct returns from wheat and that of indirect returns from sidelines, submitted a strong case for the abandonment of wheat-growing as the major operation, and hence mixed farming, in these regions. The Committee concluded that the worst portions of the marginal lands "within which it is impracticable to continue with any system of land use that involves even periodic cropping for grain, must of necessity be turned over to sheep grazing (with perhaps a few cattle) on an extensive scale, as no other proposition can provide the basis for security . . ." Apart from those hundreds which were omitted from consideration in the present analysis for reasons given in Section III(a), and ten minor exceptions, of which every one can be given adequate account, it will be observed in Figure 12 that the yields of all hundreds designated as marginal by the Committee follow courses of types I and II(10) or I, II, and III(11), and this result lends material support to their conclusion. The marginal boundaries as given in Figure 12 have been chosen to conform with the territorial unit which forms the basis of this investigation and should be regarded as indicating the approximate position of the line. Wannamana, Kappakoola, Warrambo (Eyre), and Vincent, Wilson, McPherson (Pinnaroo) constitute two doubtful areas. Their mean yields were low, but their rates of increase were quite definite and constant up to 1941, and consequently additional data must be examined before final judgment is passed. The remaining exceptions have been discussed in Section V(c).

Next in order are mallee soils situated in an intermediate zone of 10-14 in. seasonal rainfall. The hundreds concerned are Ripon, Scott, Murray (Nuyts), Telowie, Yongala (Flinders), Pirie, Napperby, Wandearah, Mundoora, Whyte, Terowie, Everard (Goyder), English, Neales, Dutton, Inkerman (Light), Finnis, Mobilong, Burdett, Ettrick, and Seymour (Sturt), which, except for Everard and Inkerman, are either contiguous or nearly so with the inner marginal boundary. In these areas the decline may not supervene until after 25-35 years of cropping with an ample supply of superphosphate, and except for Pirie its magnitude is not as great relatively as in the previous cases.

Thirdly, follow the heavier loamy mallee soils with a rainfall of 12 in. or more, and the red brown earths in Flinders, Goyder, Yorke, and Light. The dominant form of trend in this group of 78 hundreds is type IV(10). More significant observations are, however, that there are only six courses of type VI(10), each of which is characterized by special circumstances, and the presence of a type II(10) curve in Maitland, a district that has always been regarded as one of the best centres for cereal culture. The yields of barley in Maitland follow a similar course, thus confirming the result for wheat. It is of some moment also that the rate of increase in yield of the type V(10) curve of Curramulka, had very nearly reached zero by 1941. These observations, when

coupled with the widespread advance of declining yields in the submarginal zone, provide a timely and salutary warning that the existing cropping systems must be altered radically to prevent further deterioration.

Since this group constitutes the heart of the industry, from which approximately 80 per cent. of the total harvest is gathered, it is worth while furnishing additional detail regarding the trends. Table 4 sets out the rates of increase in yield at four points of the period 1896-1941, together with the yield of 1941, adjusted for rainfall. It must be recognized that the production of the soil-

TABLE 4
RATES OF INCREASE OF YIELD AT SELECTED DATES IN THE PERIOD 1896-1941

Region* and Hundred	Rate of Increase of Yield (bush./ac./an.)				1941 Yield	
	1900	1911	1921	1941	(bush./ac.)	(bush./ac./in. seasonal rainfall)
<i>Flinders</i>						
Gregory	0.43	0.19	0.14	0.09	13.89	1.21
Wongyarra	0.59	0.44	0.32	0.06	21.37	1.31
Booleroo	0.53	0.25	0.18	0.12	16.58	1.38
Pekina	0.44	0.21	0.16	0.11	13.50	1.27
Black Rock Plain	0.40	0.21	0.16	0.11	12.18	1.27
Appila	0.57	0.26	0.19	0.13	18.22	1.34
<i>Goyder</i>						
Tarcowie	0.56	0.27	0.19	0.14	17.20	1.41
Mannanarie	0.50	0.22	0.16	0.11	15.81	1.39
Howe	0.61	0.31	0.23	0.16	18.37	1.35
Booyoolie	0.64	0.31	0.23	0.16	19.46	1.34
Caltowie	0.56	0.26	0.19	0.13	17.46	1.31
Yangya	0.65	0.30	0.22	0.15	20.44	1.55
Belalie	0.56	0.24	0.17	0.11	18.96	1.33
Crystal Brook	0.49	0.21	0.15	0.10	15.99	1.26
Narridy	0.73	0.39	0.30	0.22	22.14	1.73
Bundaleer	0.86	0.46	0.35	0.26	25.84	1.74
Reynolds	0.52	0.22	0.15	0.10	18.81	1.37
Anne	0.60	0.26	0.18	0.12	19.92	1.45
Hallett	0.96	0.28	0.14	0.06	17.30	1.32
Red Hill	0.50	0.22	0.16	0.10	16.58	1.33
Koolunga	0.57	0.27	0.20	0.14	17.54	1.42
Yackamoorundie	0.64	0.29	0.21	0.14	20.15	1.59
Andrews	0.67	0.31	0.22	0.15	21.35	1.61
Ayers	0.97	0.20	0.09	0.03	17.73	1.29
Kingston	0.96	0.25	0.12	0.05	17.19	1.27
Wokurna	0.44	0.20	0.14	0.10	13.96	1.15
Barunga	0.58	0.28	0.21	0.14	17.73	1.36
Boucaut	0.80	0.17	0.07	0.03	14.90	1.18
Hart	0.73	0.36	0.26	0.19	22.12	1.74
Milne	0.65	0.28	0.20	0.13	21.77	1.28
Hanson	1.09	0.30	0.15	0.06	19.49	1.31
Koorunga	1.03	0.31	0.15	0.06	18.60	1.28
Cameron	0.49	0.22	0.16	0.11	15.73	1.34
Blyth	0.47	0.19	0.13	0.09	16.58	1.21
Clare	0.46	0.36	0.27	0.09	21.09	1.03

* The name of the region is printed in italics.

TABLE 4 (*continued*)

Region* and Hundred	Rate of Increase of Yield (bush./ac./an.)				1941 Yield	
	1900	1911	1921	1941	(bush./ac.)	(bush./ac./in. seasonal rainfall)
<i>Light</i>						
Goyder	0.48	0.24	0.17	0.12	14.50	1.32
Stow	0.55	0.09	0.04	0.01	11.67	0.97
Hall	0.60	0.28	0.20	0.14	18.66	1.39
Upper Wakefield	0.64	0.28	0.20	0.14	20.63	1.21
Stanley	0.70	0.32	0.23	0.16	22.34	1.32
Apoinga	0.97	0.33	0.17	0.07	18.05	1.25
Saddleworth	0.68	0.30	0.22	0.15	21.95	1.40
Waterloo	0.60	0.27	0.19	0.13	19.29	1.19
Balaklava	0.46	0.20	0.15	0.10	14.60	1.16
Dalkey	0.55	0.24	0.17	0.12	17.72	1.37
Alma		0.34			24.09	1.66
Gilbert	0.65	0.30	0.21	0.15	20.68	1.27
Julia Creek	0.51	0.22	0.16	0.10	17.15	1.15
Dublin	0.42	0.18	0.13	0.09	14.00	1.05
Grace	0.51	0.22	0.15	0.10	17.11	1.28
Port Gawler	0.39	0.17	0.12	0.08	13.05	1.01
Mudlawirra		0.30			20.41	1.42
Light	0.54	0.23	0.16	0.11	17.85	1.18
Nuriootpa		0.20			16.56	1.10
Kapunda	0.54	0.24	0.17	0.11	17.57	1.15
Belvidere	0.75	0.16	0.07	0.03	13.64	0.82
Moorooroo	0.20	0.19	0.18	0.17	15.30	0.93
Jellicoe	0.36	0.15	0.10	0.07	12.99	0.89
<i>Yorke</i>						
Tickera	0.49	0.26	0.19	0.14	14.83	1.32
Wiltunga	0.53	0.27	0.20	0.14	16.08	1.24
Kadina	0.52	0.25	0.18	0.13	16.12	1.25
Ninnes	0.59	0.30	0.23	0.16	17.78	1.43
Kulpara	0.56	0.26	0.19	0.13	17.63	1.37
Tiparra	0.68	0.38	0.30	0.22	20.61	1.55
Clinton	0.55	0.28	0.21	0.15	16.56	1.27
Kilkerran	0.98	0.28	0.14	0.05	17.60	1.44
Maitland	0.72	0.51	0.32	— 0.06	21.45	1.28
Cunningham	0.63	0.35	0.27	0.20	18.98	1.52
Wauraltee	0.62	0.31	0.24	0.17	18.62	1.34
Muloowurtie		0.46			22.69	1.71
Koolywurtie	0.56	0.26	0.18	0.13	17.75	1.17
Curramulka	0.64	0.48	0.33	0.03	21.12	1.58
Minlacowie	0.44	0.35	0.27	0.11	19.79	1.38
Ramsay	0.52	0.25	0.18	0.13	16.06	1.09
Para Wurlie	0.65	0.48	0.32	0.00	20.11	1.33
Moorowie	0.60	0.29	0.22	0.15	18.20	1.20
Dalrymple	0.37	0.32	0.27	0.18	18.80	1.30
Melville	0.31	0.30	0.29	0.28	19.29	1.37

* The name of the region is printed in italics.

rainfall combination is limited, so that ultimately yield must slowly approach an asymptote. Comparison of the 1941 yields in Table 4, expressed as bushels per acre per inch seasonal rainfall, with the known potentialities of the most recent varieties at that time, which under favourable field conditions would yield $1\frac{1}{2}$ to $2\frac{1}{2}$ bushels per acre per inch, shows clearly that the retardation is due to causes other than those imposed by natural limitations. It will be observed that only 11 hundreds exceed the lower limit of $1\frac{1}{2}$ bushels per acre per inch. Actually the figures given are slightly exaggerated, since no account has been taken of the amount of water conserved by fallowing. This depends on characteristics of the rainfall distribution, physical properties of the soil, and depth of penetration of the plant's root system, and in some places may exceed the equivalent of 4 in. of rain.

With these hundreds also, there can be no doubt that the nitrogen content of the soils is being depleted, as indicated by the progressive retardation in the course of yield, but their originally greater reserves and the regular appearance of improved varieties during the past 20-30 years have been major factors in preventing the occurrence of a decline in many of them. Under the better conditions of rainfall, continual use of superphosphate has encouraged the development of annual legumes, so that, in addition, symbiotic fixation of nitrogen has made appreciable contributions toward the maintenance of the nitrogen supply, but its effects cannot be assessed accurately without further information.

Finally, there are parts of southern Nuyts, Eyre, Pinnaroo, and Tatiara, the principal soils being sandy and loamy mallee, transitional mallee-solonetz, and Wimmera grey loam. In all hundreds the trends are either type VI(10) or type IV(11), these forms being directly attributable to the advances made in the past 40 years. The important point, however, is that the effects are manifested in this manner because a large proportion of the original nitrogen reserves still remains owing to the comparatively recent development of the areas. The Wimmera grey loam is a special case, noted for its high organic matter content, and constituting one of the most fertile wheat soils of Australia. The progress made is remarkable, despite the exploitative nature of the cropping system, and to date there is no conclusive evidence of physical deterioration. Of the remaining three soil types, the only one represented in places where cropping began prior to 1896 is loamy mallee. The marked contrast between the forms of trend in Figures 24 (Narriby), 29, 30 (Curramulka), and Figures 19, 30 (Muloowurtie) is largely an expression of this difference, the longer cropping history of the hundreds in the first group having led to a greater depletion of the nitrogen reserves. The experiences gained on the older areas provide a warning for the four soil types, particularly sandy mallee and transitional mallee-solonetz, as these are more likely to be affected first.

(iv) *Restoration and Maintenance of the Nitrogen Status.*—In these regions, with their comparatively reliable seasonal rains, the economic restoration and maintenance of the nitrogen supply can be effected by lengthening rotations to include several years of forage crops and/or temporary pasture which embody

a legume and are capable of supporting maximal numbers of livestock. At the same time, this system periodically rebuilds soil structure.

A classical illustration is provided by the analysis of an accurate record of yield taken near Saddleworth. The property concerned passed into the hands of the present owners in 1897, after having been worked on a fallow-wheat rotation, probably for many years. In 1897 a three course rotation, fallow-wheat-pasture, was adopted and retained until 1924. The pasture phase of this rotation was replaced by oats in 1925, and shortly after, three longer rotations were introduced, namely,

fallow-wheat-oats-fallow-wheat-oats (grazed)-wheat (second fallow replaced occasionally by peas),

fallow-wheat-peas-wheat-oats-pasture,

fallow-wheat-oats-pasture,

in which the pasture contained burr clover as a constituent species. Sheep and cattle have been carried on the property since 1897, and superphosphate was first applied in 1902.

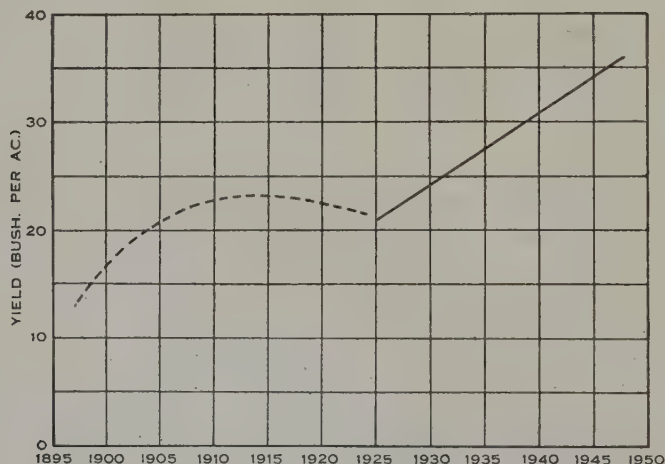


Fig. 37.—Analysis of wheat yield on a property near Saddleworth.

The sequence of yields was broken at 1924, and the two sections 1897-1924 and 1925-1948 analysed separately. Figure 37 illustrates the course of yield after allowance has been made for variations in the seasonal rainfall. The first phase shows a marked increase in yield due mainly to the use of superphosphate and improved varieties such as Federation and Yandilla King, followed by a slight fall over the 10 year period terminating in 1924, while the second shows a linear increase at the rate of $\frac{2}{3}$ bushel per acre per annum.

Cropping prior to 1897 had made phosphorus the limiting factor and reduced nitrogen reserves. As the phosphorus status was built up, consumption of nitrogen increased, and after 1915 it shows signs of becoming the limiting factor. Apart from re-arrangement of the rotations, the management has taken, since 1925, other important steps to improve the standard of farming. The total gain

of 15 bushels per acre over the second phase is partly due to these factors, principally higher-yielding varieties, but the increase in fertility attributable to the new cropping systems cannot be doubted.

Owing to the extreme difficulty of securing long and accurate records such as this, it is impossible to obtain confirmation of these results over a range of large-scale field conditions. The only data suitable for analysis are the reported results of rotation trials that have been conducted in Victoria and South Australia.

The observations used by Forster (1939) were derived from experiments laid down by the Department of Agriculture, Victoria, at three widely separated centres, Longerenong, Werribee, and Rutherglen. Rainfall at the experimental sites ranges from 15 to 23 in., and the soils, in the order given, are the typical Wimmera grey loam, a basaltic alluvium, and a grey-red buckshot silty loam, none of which had been seriously depleted by cropping prior to the initiation of the trials. Superphosphate was the only manurial treatment applied to the plots. Yield of wheat from the rotation fallow-wheat, after adjustment for rainfall, was maintained at two centres, and increased at the third — Werribee. On the other hand, oat yields from the rotation fallow-wheat-oats declined markedly and significantly at the three places, indicating a definite reduction in fertility. The fact that wheat showed no evidence of this was probably attributable to the increased amounts of superphosphate and improved varieties that were incorporated from time to time in the trials. By differencing the yields of wheat from rotations involving one year's grazing and fallow only, Forster showed that yield from the former type of rotation was gaining significantly on the latter at Rutherglen and Werribee, but not at Longerenong. This would indicate that fertility was increased at the first two centres under the rotations used, since wheat yields were either maintained or increased at these places in the fallow-wheat rotation.

At Rutherglen, the responses to temporary sown pasture of the rotations was much greater than that obtained at Werribee and Longerenong, where the corresponding phase was a year's ley. Forster stated that other trials at Rutherglen in which pasture was ploughed in have shown a gain in fertility, thus demonstrating that with longer periods of pasture in the rotation, greater improvement in fertility can be expected.

Wark (1942), following the method outlined by Forster, analysed yield data from trials conducted at Booborowie, the Waite Agricultural Research Institute, and Roseworthy, in South Australia. The soil at the first two centres is a red brown earth, and at the third a loamy mallee, and seasonal rainfall is, in the order given, 14.4, 20.1, and 14.6 in. At the Waite Institute the trial was begun on virgin land, but the previous history of the experimental sites at the other two centres is not known definitely, though it is certain that cropping was conducted on each for some years.

The only data available at Roseworthy were yields derived from a fallow-wheat rotation under several manurial treatments, and in each, yield declined significantly, even in the presence of ample supplies of superphosphate.

At Booborowie, wheat yields were maintained in the fallow-wheat rotation, but yields of stubble-sown oats and barley, from fallow-wheat-oats and fallow-wheat-barley rotations, respectively, declined strongly and significantly, showing as with Forster's data, a reduction of fertility. By differencing yields of wheat from rotations incorporating either one year of natural pasture or two years of ryegrass pasture, and from the rotation fallow-wheat, it was found that yield was maintained. Inclusion of one year of pasture also materially reduced the rate at which stubble-sown oats was declining.

With the addition of seven years' observations to the data studied by Wark, the yields from four rotations at the Waite Institute have been re-examined. The period covered by the new analysis is 1925-47 inclusive, and the rotations concerned are

1. fallow-wheat,
2. fallow-wheat-oats,
3. fallow-wheat-barley,
4. fallow-wheat-oats-pasture (Wimmera ryegrass),

in which all crops received a dressing of 187 lb. per acre superphosphate. Wheat yields declined in the first three rotations, and increased in the last, but owing to the excessive annual variation, none of these results was statistically significant, even after making allowance for fluctuations in seasonal rainfall.

When differences between yields of wheat in the series were taken, the following results were obtained:

Difference of Wheat Yield	Regression Coefficient (bush./ac./an.)
fallow-wheat-oats-pasture — fallow-wheat	0.27
fallow-wheat-oats-pasture — fallow-wheat-oats	0.58
fallow-wheat-oats-pasture — fallow-wheat-barley	0.28

but, as before, after adjusting for rainfall, annual variation still masks other effects, and only the second coefficient is significant, though the other two have the sign which was expected. The mean yields of wheat in the four rotations over the period were

Rotation	1	2	3	4
Mean yield (bush./ac.)	36.2	35.8	37.5	44.9

from which the superiority of the four-course rotation is evident, since the 7.9 bushel differences are very significant.

Finally, pending a more detailed analysis, a preliminary examination has been made of a rotational trial embodying a modern experimental design. The five rotations are

1. fallow-wheat,
2. fallow-wheat-peas-pasture (Wimmera ryegrass),
3. fallow-wheat-subterranean clover-pasture (Wimmera ryegrass),
4. fallow-wheat-oats-pasture (Wimmera ryegrass)
5. fallow-wheat-barley-pasture (Wimmera ryegrass).

All crops, including the pasture, received superphosphate at 2 cwt. per acre during 1937-41, but this was reduced to 1 cwt. per acre in subsequent years, since the phosphate supply had been built up during an interval prior to the beginning of the experiment. Half of each plot sown to a forage crop, namely, peas, clover, oats, and barley, was harvested each year and the produce removed, while the other half was grazed *in situ*. For present purposes, it is sufficient to quote the mean yields at the termination of the eighth year. These are set out in Table 5.

TABLE 5
MEAN YIELDS OF ROTATION TRIAL AT WAITE AGRICULTURAL RESEARCH INSTITUTE

Rotation	1	2		3		4		5	
		Grazed	Harvested	Grazed	Harvested	Grazed	Harvested	Grazed	Harvested
Mean yields of wheat (bush./ac.)	31.87	43.49	41.59	37.41	34.32	39.77	39.14	37.74	36.19
		42.54		35.87		39.46		36.96	
Mean yields of forage crop total produce (air-dry) (cwt./ac.)		46.11	39.80	26.04	25.47	19.25	20.85	20.42	22.84
		42.96		25.76		20.05		21.63	
Mean yields of pasture (oven-dry) (cwt./ac.)		51.35	33.43	23.81	19.07	14.44	10.71	16.84	11.50
		42.39		21.44		12.57		14.17	

The yield of wheat from the fallow-wheat rotation is significantly lower than that of all four-course rotations, and there are significant differences among the latter, the most important being that the rotation including peas outyields the remainder. The pea crop is the outstanding forage, with a mean yield significantly greater than clover, oats, and barley. Two dry seasons, 1938 and 1940, greatly reduced the yield of subterranean clover as compared with peas, and this partly accounts for the large difference in the general means, but the mean yield of clover is significantly greater than that of barley or oats. Marked differences in the yield of temporary pasture in the various rotations have been evident throughout, and they have increased with time, all being significant. The effect of grazing is significant in the four cases.

Insufficient time has elapsed to show appreciable effects of the legumes on wheat yields in comparisons among the four-course rotations, but their superiority in other phases is clearly defined. The great advantage of peas compared with clover in this environment is also well established.

The high yields of wheat from the fallow-wheat rotation in this trial, 31.9 bushels per acre, and 36.2 bushels per acre in the one quoted above, are indicative of the ability of this particular soil type to withstand the strain of frequent cropping.

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THE METABOLISM OF THE APPLE DURING STORAGE

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Summary

A survey has been made of changes in amounts of certain metabolites and possible respiratory intermediates in the flesh of Australian Granny Smith apples during storage at 0°C.

Respiratory activity shows two peaks and carbohydrates form the predominant substrate for respiration. Evidence indicates that a carbonyl compound of low molecular weight may be an intermediate in carbohydrate metabolism. Fluctuations in total organic acids, malic acid, and citric acid suggest that the tricarboxylic acid cycle of Krebs may operate in carbohydrate oxidation in the apple. Ascorbic acid, which decreases during storage, and oxalic acid, which remains constant, do not fluctuate significantly with the respiration rate. Total nitrogen remains approximately constant during storage but there is an appreciable synthesis of protein. This synthesis appears to be related to the large reserves of available carbohydrate and is dependent on the level of respiratory activity.

I. INTRODUCTION

Although a number of chemical and physiological studies on the apple have been carried out, there is no detailed knowledge of the respiratory mechanisms of the fruit even though such information is of fundamental interest and of considerable practical importance. Several workers (e.g. Haynes and Archbold 1928; Onslow, Kidd, and West 1931; Hulme 1932) have followed chemical changes in the apple during storage and this approach has been extended in the present investigation by carrying out a comprehensive survey of changes in a number of substances, including respiratory substrates and possible intermediates. Similar techniques, using starving leaves (e.g. Vickery *et al.* 1939; Wood, Cruickshank, and Kuchel 1943) have proved successful in providing an integrated metabolic scheme for certain leaf tissues, and the formulation of such a scheme for the apple is a desirable preliminary to a detailed investigation of the biochemistry of the fruit.

The Granny Smith apple, a variety of major commercial importance in Australia and the subject of previous physiological work in this laboratory (e.g. Trout *et al.* 1942), was used in this investigation. The present paper describes studies on flesh tissues only.

II. EXPERIMENTAL

(a) *Material and Method*

A selected Granny Smith apple tree at Orange, New South Wales, was stripped when the crop was at normal commercial maturity, and unblemished

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fruit from three adjacent size classes placed in a storage room maintained at 0°C., the temperature usually employed in commercial practice. The apples were wrapped in oiled paper (to prevent superficial scald), kept in darkness, and an adequate air circulation around them was maintained. Samples, each consisting of 30 fruits with proportional representation from each size class, were taken at random at approximately monthly intervals for determination of respiration rate and for subsequent chemical analysis. The mean weight of each sample of 30 fruits was approximately constant at the beginning of storage; in all, 15 samplings were taken. The analyses carried out included starch, alcohol-insoluble residue, reducing sugars, non-reducing sugars ("sucrose"), total organic acids, malic acid, citric acid, oxalic acid, total carbonyl compounds, ascorbic acid, total nitrogen, and protein nitrogen. Changes in fresh and dry weight were followed.

All the fruits were initially free from blemish, skin injury, and mould infection. During the later stages of storage life (after 320 days in store) approximately one-third of the fruit was suffering from "lenticel spot," which is a non-parasitic disorder confined to the skin tissue and is unlikely to have any effect on the respiration rate of the flesh. In the later storage samples, up to 10 per cent. of the fruit was suffering from mould infection. The affected fruits were replaced with sound specimens of similar initial weight from the same storage population before any determinations were carried out; it was not considered advisable to prolong the experiment further as wastage became increasingly severe. The significance of the random sampling would then be doubtful, with the population consisting only of the longer-lived individuals. These may differ in respiration rate and chemical composition from the random population and it is difficult to assess the probable extent of such a variation.

(b) Analytical Methods

Fresh Weight.—Changes in fresh weight were followed by weighing the whole fruit. Each fruit was weighed separately at the beginning of storage, placed in a labelled wrap, and weighed again when taken for sampling. As the weight of the non-flesh tissue of the Granny Smith apple is a relatively small proportion (approximately 5.6 per cent.) of that of the whole fruit, it is considered that this method gives a sufficiently accurate indication, for the purposes of this experiment, of the changes in fresh weight of the flesh region. The changes in dry weight have been calculated on this assumption.

Dry Weight.—The fruits were peeled, cored, and quartered, care being taken to ensure complete removal of all chlorophyll-containing tissue from the outer cortex and all carpel wall tissue etc. from the pith. The flesh from opposite quarters was grated at 0°C. and aliquots of the grated material dried in a draught-oven at 70°C. for 3-4 hours and then in a vacuum oven at 70°C. for 6 hours.

Respiration Rate.—The rate of carbon dioxide production was determined on whole fruits by drawing a stream of CO₂-free air over the samples enclosed in glass containers. The entire sample of 30 apples was used. From three to five

readings, spread over three days, were taken for each sample. The respired carbon dioxide was estimated by the Pettenkofer method by absorption in $0.2N$ $Ba(OH)_2$ and titration with $0.2N$ HCl , using phenolphthalein.

Carbohydrates.—Opposite peeled quarters of the fruit were grated and 100 g. of this fresh grated material blended in a Waring Blendor with 100 ml. of water. Aliquots of the blended suspension were removed immediately for the determination of starch and sugars.

Starch.—The starch in the aliquots was subjected to preliminary solubilization with perchloric acid and estimated colorimetrically by the method of Nielsen (1943), as modified by Nielsen and Gleason (1945). Light absorption due to the starch-iodine complex was measured at 6600\AA . The method was standardized with a pure starch prepared from immature Granny Smith apples.

Sugars.—Aliquots of the blended material were diluted with water and heated in a boiling water bath in the presence of calcium carbonate (to prevent hydrolysis of the sucrose present). The suspension was cleared with zinc hydroxide and reducing sugars estimated using the copper reagent of Somogyi (1937). The increase in reducing power after hydrolysis with $0.1N$ HCl at $100^\circ C$. was termed "sucrose." It has been found in this laboratory (F. E. Huelin 1945, unpublished data) that non-sugar-reducing substances in apple flesh amount to only 1.3 per cent. of the total sugars. For practical purposes, it may be assumed that only sugars react in the above method.

Ascorbic Acid.—Slices of flesh were cut, dropped immediately into 5 per cent. metaphosphoric acid, and blended in the Waring Blendor. Ascorbic acid was determined in filtered aliquots of the blended material by titration with 2, 6-dichlorophenol-indophenol.

Total Carbonyl Compounds.—Fresh grated material was blended at $0^\circ C$. with 10 per cent. trichloroacetic acid and filtered. Determinations of total carbonyl compounds in the filtrate were made by the direct method of Friedemann and Haugen (1943), light absorption by the resultant 2, 4-dinitrophenylhydrazones in alkali being measured at 4000, 4200, 5200, and 5400\AA .

Total Nitrogen.—Peeled opposite quarters of the fruits were sliced and then dried in the draught and vacuum ovens as described in the determination of dry weight. The dried material was finely ground and used in all the following estimations. Total nitrogen was determined in dried tissue by a modified micro-Kjeldahl method.

Protein Nitrogen.—Dried tissue was extracted for eight hours in a Soxhlet apparatus with 75 per cent. alcohol. This solvent has been shown by Hulme (1932) to effect a very good extraction of non-protein nitrogen from apple flesh. The nitrogen in the extracted residue was estimated by digestion as for total nitrogen.

Alcohol-insoluble residue was estimated by drying and weighing the residue after the extraction of the non-protein nitrogen with alcohol.

Organic Acids.—The preparation of an organic acid extract was carried out by extraction of dried tissue with peroxide-free ether as described by Pucher,

Vickery, and Wakeman (1934a), using the modifications of Pucher, Wakeman, and Vickery (1941).

Total organic acids were determined in the organic acid extract by a modification of the titrimetric method of Pucher, Wakeman, and Vickery (1941). It has been found that interference by sulphuric acid (which is used to acidify the dried tissue prior to ether extraction and which is itself partly extracted) can be avoided by the addition of excess barium nitrate. The separate gravimetric estimation of sulphuric acid and the application of an appropriate correction factor as described by Pucher, Wakeman, and Vickery (1941) can thus be avoided and the time taken for the determination considerably decreased. The following method has been found suitable:

2 ml. 0.5N barium nitrate, 2 ml. 1N nitric acid, and 3 drops of brom-cresol purple are added to 10 ml. of the organic acid fraction contained in a tall 50 ml. beaker with a graduation mark at 25 ml. The solution is boiled for 1 minute, cooled, made alkaline with 1N NaOH (CO_2 -free), and adjusted to pH 8.0 with 0.05N nitric acid. The titration to pH 2.60 is carried out with 0.05N HNO_3 as described by Pucher, Wakeman, and Vickery (1941). The method is standardized with a pure sample of malic acid.

The presence of barium nitrate itself has no significant effect on the titration, but the non-removal of sulphuric acid can introduce errors in excess of 25 per cent.

Malic acid was estimated by a modification of the method of Pucher, Vickery, and Wakeman (1934b). Light absorption was measured at 5800 Å.

Citric acid was determined by the method of Speck, Moulder, and Evans (1946), using *n*-hexane as solvent for the pentabromacetone.

Oxalic acid was estimated by the method of Pucher, Wakeman, and Vickery (1941).

All the analytical data are expressed as g. (unless stated otherwise) per 100 g. of the dry weight at the beginning of storage.

(c) Statistical Methods

Analysis of variance has shown that the differences in respiration rate between various times in storage (Fig. 1) are too great to be accounted for by sampling and/or experimental error. It was found that the trend for respiration rate could be represented by a quadratic regression on time and the deviations in respiration rate from this regression line were then correlated with the corresponding deviations of the metabolites from their respective quadratic regression lines on time. The method adopted followed the original procedure of Fisher (1924), using the modified computation of Davis and Pallesen (1940). This method requires that in correlating two series of observations both dependent on time, the same power of regression correction on time should be applied to both series.

The deviations from the quadratic regression lines were subjected to analysis by the method of Kendall (1946) to determine if these residuals could be regarded as a random set. The small number of observations in any one series, however, preclude any conclusion being drawn.

The coefficients of correlation obtained between respiration rate and certain of the metabolites are given in the appropriate sections.

III. RESULTS AND DISCUSSION

(a) *Respiratory Activity*

(i) *Introduction*

It has been found in this laboratory that Granny Smith apples stored at low temperatures (0-5°C.) have a respiratory quotient closely approximating to 1, so that determination of carbon dioxide production is a reasonably accurate measure of respiratory activity and oxygen consumption. Although ethyl alcohol may be present in very small amounts, alcoholic fermentation does not take place to any appreciable extent in apples stored at these temperatures, and in the present experiment it may be assumed that aerobic conditions hold throughout the course of storage life, even during advanced senescence.

Respiratory activity has been estimated by using whole fruits, as accurate determinations on the flesh tissue alone are impracticable. The total CO₂ production of the whole fruit during the period from day 28 (when sugar concentration was at its maximum) until day 361 was 29,400 mg. per 100 g. dry weight. From the analytical data on the flesh tissue, assuming a similar rate of loss holds throughout the fruit, it can be calculated that the CO₂ output due to losses in sugars, alcohol-insoluble residue (calculated as C₆H₁₀O₅), and organic acids (calculated as malic acid) was 28,460 mg. per 100 g. dry weight or 97 per cent. of the total CO₂ production. As the weight of the non-flesh tissue is only 5.6 per cent. of that of the whole fruit, it is thought, on the basis of the above evidence, that the respiration rate determined with the whole fruit is a reasonably accurate estimate of the rate in the flesh region itself.

This interpretation, which does not agree with the suggestions of Hackney (1946) that the respiration of apple skin tissue alone may account for one-third to one-half of the CO₂ production of the whole fruit, is supported by the following experiments. A sample of 24 fruits was taken immediately after picking, when the internal oxygen concentration was high and would not limit the respiration rate. Respiration readings at 20°C. were taken separately on each fruit over a period of 4 days. After this period, during which the rate remained steady, the skin was carefully removed from 12 fruits with a sharp razor. Subsequently, the respiration rate of the whole fruits remained steady. That of the peeled apples (with the exception of four which showed evidence of fungal infection and were rejected) rose rapidly at first (perhaps due to wounding) but fell after 1-2 days to a value almost exactly that of the same fruit before peeling, and therefore remained constant for 7 days, when the experiment was concluded. Further investigations in this laboratory (M. J. Wilkins, unpublished data), with Granny Smith apples after 5 months in storage at 0°C., have also demonstrated that the respiration rates of peeled fruits are very close to those of the whole apples; in these experiments the high initial respiration rate after peeling was not so pronounced. The skin tissue removed from these fruits showed a very high initial respiration rate which fell rapidly during the next 4-5 days.

On the basis of these experiments, it has been concluded that, although the respiration rate per unit weight of skin tissue is higher than that of the flesh, it does not make such a large contribution to the total respiration of these apples as the results of Hackney would suggest, and that it is sufficiently accurate for the purposes of present investigation to accept major fluctuations in the respiration rate of the whole fruit as being largely due to changes in the respiration rate of the flesh region. In view of the observations on wounding effects and their possible implications in relation to the respiration of skin and flesh slices, it seems desirable that a more critical investigation of the respiratory rates of skin and flesh should be carried out.

(ii) Respiration Rate Changes

The form of the respiration rate/time graph in this experiment is shown in Figure 1. During the initial weeks of storage there is a fall in respiration rate followed by a small rise at day 81. A steady fall in rate then occurs with a minimum at day 165; this is followed by a rise with a maximum about day 228, another falling trend, and a final pronounced rise with a maximum at day 333. This is the highest level of respiration attained during the period of estimation

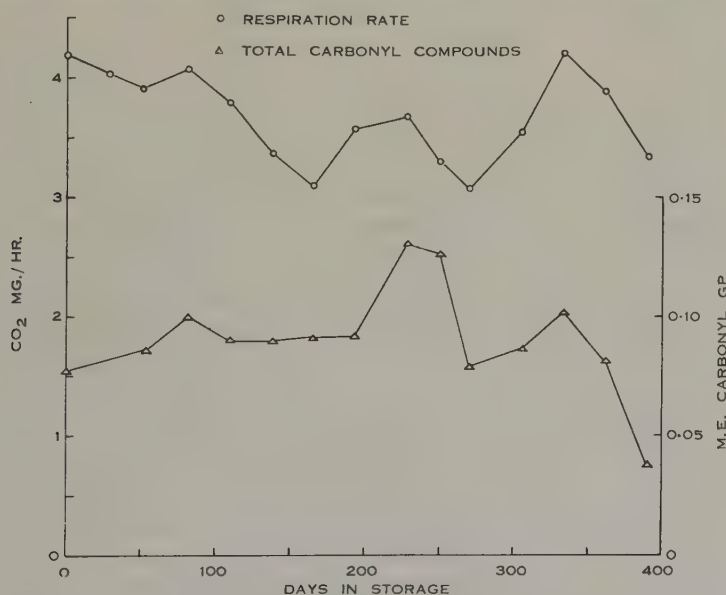


Fig. 1.—Changes in respiration rate and total carbonyl compounds during storage at 0°C. The concentration of the carbonyl compounds is *arbitrarily* expressed in terms of milli-equivalents of carbonyl group: absorption was measured at 5200 Å.

and the rate thereafter drops rapidly until the experiment is concluded. The trend, if any, within each series of readings was in good general agreement with that prevailing at the time in the graph of the mean values (Fig. 1).

The reasons for a mature resting organ, such as the apple, not subjected to external stimuli and having an abundant substrate supply, exhibiting sudden

changes in respiratory activity are obscure. Sugars, which are presumably the initial source of respiratory substrate, are at a high concentration throughout storage (even after 361 days the total sugar concentration is over 72 per cent. of the actual dry weight) and do not fluctuate significantly with the respiration rate. The respiratory rises may be caused by an increase in the activity of one or more of the enzymes involved in carbohydrate metabolism and CO_2 production or by "decrease in organization resistance" (Blackman and Parija 1928).

In most investigations with apples only one respiratory peak has been found (Kidd and West 1930; Onslow, Kidd, and West 1931) and the climacteric has been shown to be associated with the production of ethylene by the fruit (Kidd and West 1932; Gane 1935). If ethylene is the only cause of rises in respiration rate, it must be assumed that in these apples the concentration increases and decreases several times during storage, which would be unusual. The experiments of Kidd and West (1945) showed that ethylene is produced in greatly increased quantities both during and after the climacteric phase so that the normal climacteric is irreversible.

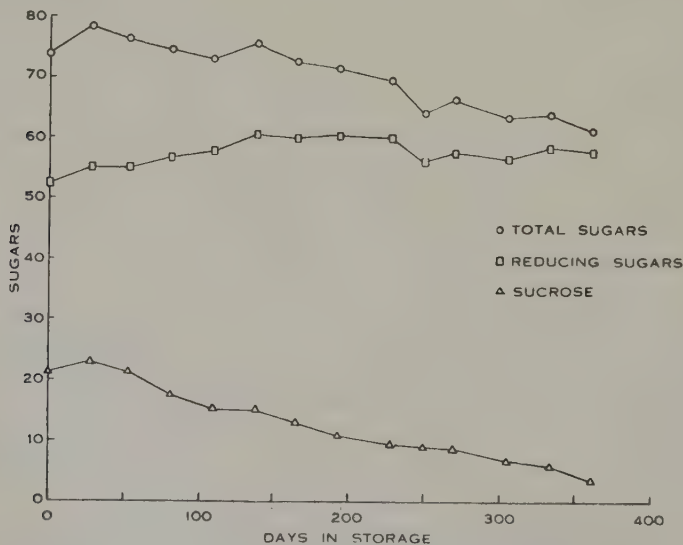


Fig. 2.—Changes in total and reducing sugars and sucrose during storage at 0°C .

(b) Carbohydrates

The changes in total and reducing sugars and "sucrose" during storage are shown in Figure 2. Total sugars, after reaching a peak of 78.4 per cent. on day 28, show a general fall to 61.3 per cent. at day 361. Reducing sugars rise from an initial value of 52.5 per cent. to 60.5 per cent. at day 138 and thereafter fall only slightly to a final value of 57.7 per cent. Sucrose rises initially to 23.1 per cent. at day 38 and then decreases steadily throughout storage: at the conclusion of the experiment it had practically disappeared.

At the commencement of storage 0.49 per cent. of starch was present: this had completely disappeared by day 28 and its hydrolysis may partially account for the initial rise in total sugars. As shown in Table 1, there is a fall of 1.2 per cent. in the alcohol-insoluble residue during this period so that components of this fraction, other than starch, may contribute to sugar formation. This decrease in alcohol-insoluble residue cannot account for all the observed rise in sugar and this is in agreement with the results of Krotkov and Helson (1946) who conclude that the initial increase in sugar when MacIntosh apples are placed in storage must, to some extent, come from some alcohol-soluble substance.

TABLE 1
CHANGES IN ALCOHOL-INSOLUBLE RESIDUE DURING STORAGE

Storage (days)	Alcohol-insoluble Residue (% dry wt.)	Storage (days)	Alcohol-insoluble Residue (% dry wt.)
0	14.2	228	12.8
28	13.0	250	12.5
53	13.3	270	11.7
81	13.1	305	12.2
109	12.9	333	11.7
138	12.3	361	11.9
165	12.6	390	11.8
193	12.5		

At low temperatures Granny Smith apples have a respiratory quotient of 1, indicating that carbohydrates are the principal substrates for respiration. This is confirmed by the analytical data. The total estimated loss of utilizable substrates in the flesh during the period from day 28 to day 361 is 28,240 mg. CO₂ per 100 g. dry wt., and of this amount, losses due to sugars (25,100 mg. CO₂) and alcohol-insoluble residue composed largely of substances which could give sugars on degradation (1800 mg. CO₂), account for 26,900 mg. CO₂, or 95.4 per cent. of the total estimated losses.

It will be seen from Table 1 that the alcohol-insoluble residue decreases steadily during storage. The main components of this fraction are probably celluloses and pectic components. Carré (1925) has made a detailed study of the changes in the pectic constituents of Bramley's Seedling apples stored at 1°C. and observed a decrease in the total pectic constituents, pectose, and especially the pectic constituents of the middle lamella. Carré assumes that the neutral pectin breaks down to pectinic acids and ultimately to pectic acid. Further breakdown to simpler acids is probable, in which case galactose, arabinose, and methyl pentose would be set free and might subsequently be utilized in respiration. From the data of Carré, it would appear that the decrease in alcohol-insoluble residue observed in the present experiment could be fully accounted for by losses in the pectic constituents. If this material is completely respired, it is a source of substrate next in importance to the sugars, forming 6.4 per cent. of the total CO₂ production calculated from decreases in the compounds estimated.

The concentration of carbohydrate material in the apple is particularly high, as the sum of total sugar plus alcohol-insoluble residue in some samples accounts for over 91 per cent. of the actual dry weight. It seems improbable that respiration is limited at any stage by the level of carbohydrate. This is in agreement with observations on other tissues such as potato (Barker 1936) and leaves (Wood 1942; Wood and Petrie 1942) where it was found that at high sugar concentrations, the respiration rate remains constant when sugar level is varied, i.e. is independent of the carbohydrate content.

Sucrose decreases at a relatively constant rate during storage and may form the preferred initial substrate for respiration or, alternatively, may be hydrolysed to reducing sugars which may enter a glycolytic cycle. It is apparent that up to day 138 at least part of the sucrose disappearing is hydrolysed to hexoses. Towards the end of the storage life sucrose had reached a very low level (3.6 per cent.) and this may be a factor causing the decline of the fruit. Results of this experiment indicate that practically all of the decrease in sucrose over the whole period is needed to account for the observed CO_2 production.

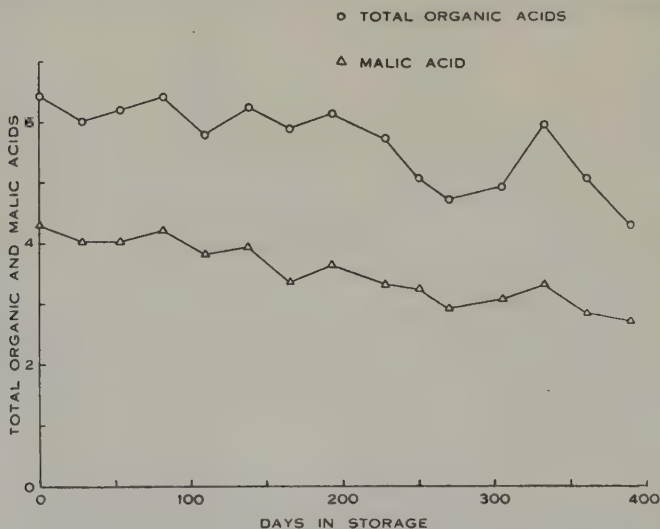


Fig. 3.—Changes in total organic acids (expressed as malic acid) and malic acid during storage at 0°C .

(c) Organic Acids

(i) Total Organic, Malic, and Citric Acids

Changes in total organic acids (expressed as malic acid) and malic acid are shown in Figure 3, and those of citric acid in Figure 4. Malic acid, in general, accounts for 60-70 per cent. of the total acid and the undetermined residue probably consists of smaller amounts of such acids as succinic, fumaric, and isocitric, together with traces of tartaric and α -ketoglutaric. Total organic acids show a tendency to decrease during storage, superimposed on this being fluctuations in the same direction as for respiration rate (Fig. 1), and correlated with it to the extent of a coefficient of 0.71, statistically significant at the 1 per

cent. level. Malic acid (much like total organic acids) exhibits a steady tendency to decrease; again the fluctuations superimposed on this tendency are in the same direction as for respiration rate and correlated with it with a coefficient of 0.73, significant at the 1 per cent. level.

Similarly for citric acid, which tends to increase from approximately the 160th day (Fig. 4), there are superimposed fluctuations which are correlated with a coefficient of 0.73 (significant at the 1 per cent. level) with the corresponding fluctuations in respiration rate.

There is a considerable body of circumstantial evidence (e.g. Vickery *et al.* 1939; Wood, Cruickshank, and Kuchel 1943) that the organic acid cycle of Krebs (cf. Krebs 1943) may play a significant part in plant metabolism. It appears that the results obtained in this investigation are consistent with the existence of the tricarboxylic acid cycle and may provide additional evidence for its operation in certain plant tissues.

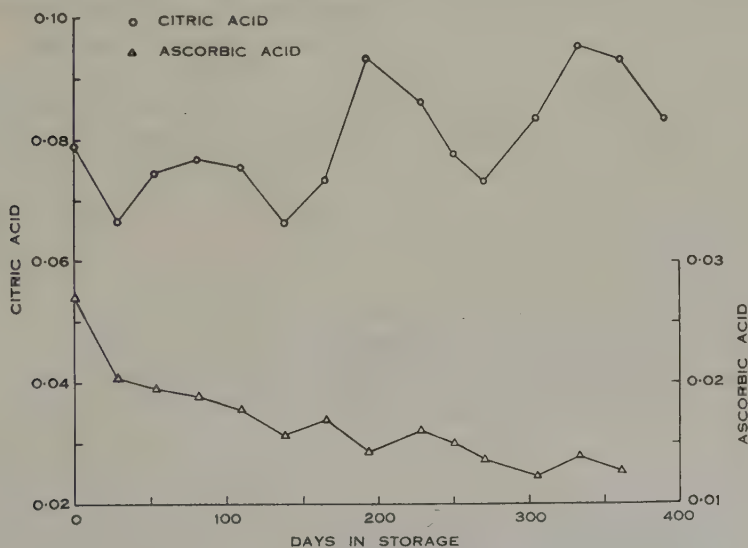


Fig. 4.—Changes in citric and ascorbic acids during storage at 0°C.

Malic acid decreases by 37 per cent. during the course of the experiment and, as no other acid product accumulates, the malic acid lost is probably completely respired to CO_2 . This would not significantly change the overall R.Q. (the calculated change being from 1.00 to 1.01). Malic acid is an intermediate of the Krebs cycle and it will be seen (Fig. 3) that at the peaks of respiratory activity this acid actually increases in concentration, presumably because of additional supplies from the acid cycle.

The fluctuations in citric acid content (Fig. 4) when considered with the curve for respiration rate (Fig. 1) indicate that this acid is intimately concerned with carbohydrate oxidation. In the later modifications of the Krebs cycle (Wood 1946), pyruvate (derived from carbohydrates) is degraded to a C_2 compound which condenses with oxalacetate to form *cis*-aconitate. This acid, under the

action of aconitase, is hydrated to both citrate and *isocitrate*, the latter being available for dehydrogenation by *isocitric* dehydrogenase and further metabolism in the Krebs cycle. In the presence of aconitase, the equilibrium between *cis*-aconitate, citrate, and *isocitrate* lies strongly in the direction of citrate formation (Johnson 1939; Krebs and Eggleston 1944) so that citric acid concentration may be used as an indication of the amounts of the other C_6 acids present, i.e. as an indicator of the amounts of these acids passing through the cycle. Thus citric acid is not an actual intermediate in the Krebs cycle but is a side reaction of aconitic acid; the ready interconversion of the three C_6 acids renders this distinction somewhat unimportant.

From the data in Figures 1 and 4, it appears that most of the total citric acid present in the tissue is actively engaged in the metabolic flux. This contrasts with the position of malic acid, which is present in much greater concentration and which also functions as a reserve substrate supply, so that the quantities actually engaged in metabolism at any one time are impossible to estimate. The correlations obtained between the organic acids and respiration rate are probably due to carbohydrate being the main substrate for respiration in the apple and to the absence of the complicating effects of protein, amino acid, and amide degradation such as arise in the study of most other plant tissues.

It can be concluded that the data of this experiment are compatible with the participation of a Krebs cycle in carbohydrate oxidation in the apple, but do not prove that such a cycle operates.

(ii) *Ascorbic Acid*

Ascorbic acid (Fig. 4) shows a pronounced fall during storage life; from an initial value of 0.027 per cent. dry weight the concentration reaches 0.013 per cent., or less than half of the original figure. This acid shows no correlation with respiratory activity and its fate is unknown.

(iii) *Oxalic Acid*

Oxalic acid is a minor acid constituent and remains very constant at 0.19 per cent. dry weight throughout storage. This is in agreement with the data of Vickery *et al.* (1939) with rhubarb leaves in culture and with narcissus plants (1946). These authors found that little change occurred in oxalic acid and that conditions of culture or starvation did not affect it in any way. The impression given is that oxalic acid is a sluggish metabolite under any conditions and it does not appear to play any dynamic role in the metabolism of the apple during storage.

(d) *Carbonyl Compounds*

The method of Friedemann and Haugen (1943) for the estimation of total carbonyl compounds involves extraction with trichloroacetic acid, incubation with 2, 4-dinitrophenylhydrazine and the addition of sodium hydroxide. Light absorption is then measured at 5200 Å and several other wavelengths. Compounds which react in this method include aldehydes, ketones, and keto-acids of relatively low molecular weight.

It has been found that phenolic substances present in the trichloroacetic acid extract of apple flesh interfere in this method. Addition of ferric chloride to the

extract gives a green colouration and addition of sodium hydroxide causes this green colour to become red owing to the formation of a complex iron salt: these reactions are characteristic for *o*-dihydroxy benzene derivatives. The red compound formed on the addition of sodium hydroxide to the ferric chloride-apple extract has a slightly different absorption spectrum from that given by catechol itself. It appears that, although the substance present in the trichloroacetic extract has the basic structure of catechol (*o*-dihydroxy benzene), there are additional groups attached to the benzene nucleus. This catechol-like substance may be identical with the chlorogenic acid isolated by Rudkin and Nelson (1947) from sweet potatoes. The phenol present is readily oxidized to an *o*-benzoquinone derivative having a reddish-yellow colour when the solution is made alkaline during the estimation of the total carbonyl compounds. The quinone so formed can react with phenylhydrazine (quinones show reactions of the carbonyl group) and can also oxidize this reagent; these products are coloured red or yellow and it is essential to apply an appropriate correction (using catechol as reference substance) before calculating the amount of carbonyl compound present. Provided the concentration of carbonyl compounds is relatively large this procedure gives satisfactory results especially if, as in the present case, the concentration of the phenol remains constant during the experiment.

The lower curve of Figure 1 shows the fluctuations in total carbonyl compounds during storage, the concentration being *arbitrarily* expressed as milli-equivalents of carbonyl groups per 100 g. dry weight. The carbonyl compounds show several interesting variations: there is a small rise at day 81, followed by maxima at day 228 and day 333 and a pronounced fall at day 390. There is a slight tendency to increase in the first 193 days, and a steady decrease from then on, superimposed on these trends being fluctuations in the same direction as for respiration rate, and correlated with it to the extent of a coefficient = 0.64, which is significant at the 5 per cent. level.

The carbonyl compounds thus appear to play some part in metabolic activity prior to the liberation of carbon dioxide and seem to be closely associated with the respiration rate rises. With each of the peaks there is a simultaneous carbonyl compound maximum, suggesting that the carbonyl compound may be an intermediate in carbohydrate breakdown as carbohydrates are responsible for practically all the CO₂ production in apple tissue. The concentration of the carbonyl compound is small — of the order of 0.1 milli-equivalents per 100 g. dry weight — but would not be unusual for an intermediate of a reactive nature. The magnitude of the role played by the carbonyl compound cannot be determined on the available evidence, but it may be important especially during the periods of increased respiratory activity.

Contrary to expectations based on the reported occurrence of acetaldehyde in apple tissue (e.g. Thomas 1925; Hulme 1933; Fidler 1936), this substance does not appear to be present in significant amounts in Australian Granny Smith apples stored at low temperatures. The reasons for this discrepancy and evidence concerning the identity of the carbonyl present in the apple flesh will be presented

in a subsequent communication. The substance is not an α -keto acid as the extraction method of Friedemann and Haugen (1943), which is relatively specific for the naturally occurring α -keto acids, did not give comparable results.

(e) Nitrogen Metabolism

Changes in total and protein nitrogen during storage are shown in Figure 5. Total nitrogen remains approximately constant during storage although there is a slight rising trend in the later stages. This, as shown below, may be due to transport of soluble nitrogen components from the skin, but it is probable that the earlier fluctuations in total nitrogen are largely due to sampling errors, as it has been found that a series of individual apples picked from the same tree may exhibit substantial variations in total nitrogen concentration; conversely, protein nitrogen shows remarkably little variation in such a series. During storage, protein nitrogen (see lower curves in Figure 5) shows a significant increase from 0.101 g. per cent. at day 53 to a maximum of 0.137 g. per cent. at day 390.

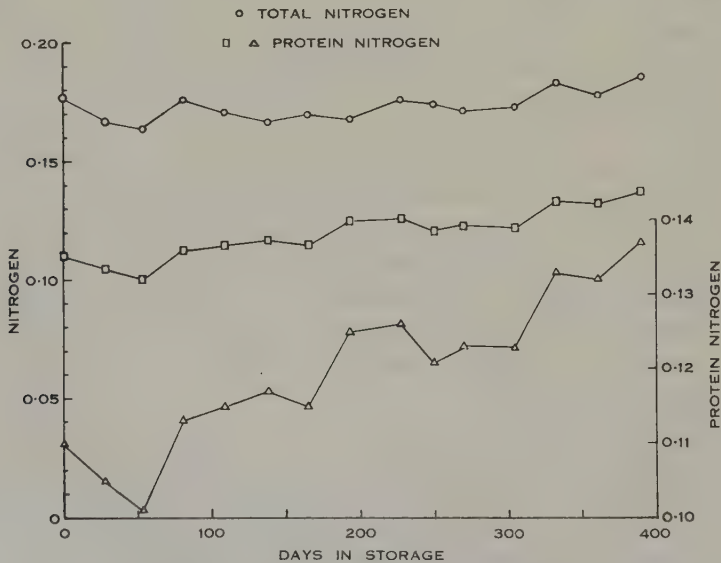


Fig. 5.—Changes in total and protein nitrogen during storage at 0°C. The lower graph for protein nitrogen is on an enlarged scale (shown at right).

Protein synthesis as a percentage of total nitrogen is shown in Figure 6, the graph for non-protein nitrogen (total nitrogen *minus* protein nitrogen) being included. Protein nitrogen, expressed as a percentage of total nitrogen, increases from 61.8 per cent. to a maximum of 75 per cent. at day 361, and, as there is no loss in total nitrogen, protein synthesis has definitely occurred.

During prolonged storage, the green colour of the skin of the Granny Smith apple changes to yellow, indicating chlorophyll breakdown. Yemm (1937) and others have noted that the onset of protein digestion in starving leaves is simultaneous with the beginning of obvious chlorophyll degeneration. It seems

probable that certain of the proteins in the skin tissue are hydrolysed and the products of proteolysis (amino acids etc.) may be transported into the cortex region and so give a higher total nitrogen figure for the flesh during the later stages of storage. It is apparent, however, from the data in Figure 6, that the rise in protein nitrogen during storage is in excess of any increase in total nitrogen in the flesh.

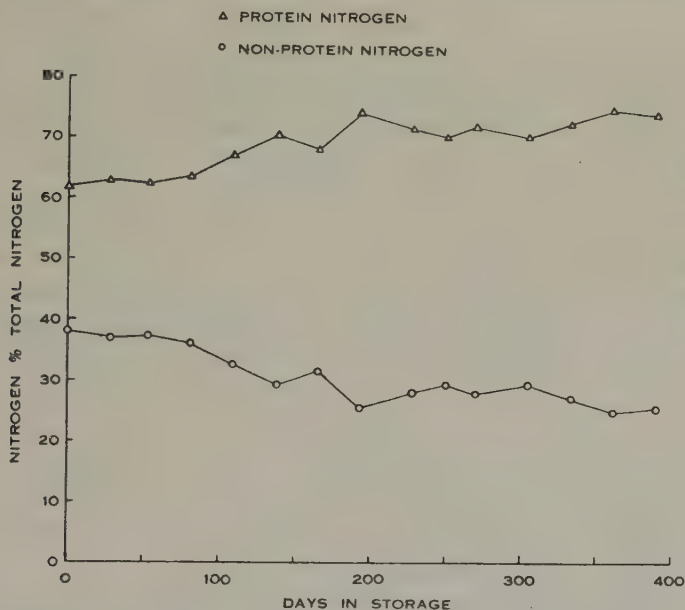


Fig. 6.—Changes in protein and non-protein nitrogen as percentages of total nitrogen during storage at 0°C.

Hulme (1932) has observed a rise in protein nitrogen in Bramley's Seedling apples in storage at 1°C. and the apple appears to be one of the few starving plant tissues which synthesize protein while in an adult resting state. When a leaf is detached from a plant, digestion of protein, with increase in soluble nitrogen, soon becomes apparent (e.g. Vickery *et al.* 1939); an exception to this is found in an experiment of Wood, Cruickshank, and Kuchel (1943) where proteolysis was considerably delayed owing to a high concentration of sucrose in the particular sample. Protein degradation did not occur until this reserve was considerably decreased. A similar observation with leaves having large stores of starch and other carbohydrates was made by Deleano (1912) who found no loss of protein until after 100 hours in culture. The apples used in this experiment provide a similar example in that carbohydrate reserves are very large, total sugars always being greater than 60 per cent. of the original dry weight. A possible explanation of this "protein sparing effect" is that when carbohydrate content is high, carbohydrate degradation products, which are the starting point for amino acid synthesis, are formed in high concentration and are converted into amino acids at a rate greater than the oxidation of the latter. In the present investigation, the three main rises in protein nitrogen (Fig. 5),

which account for most of the total observed increase, occur at days 53, 165, and 305 and coincide with periods of markedly increasing respiratory activity (Fig. 1) so that protein nitrogen level and respiration rate appear to be related. The problem of protein metabolism in the apple will be considered in greater detail in a subsequent publication.

IV. ACKNOWLEDGMENTS

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ON THE MECHANISM OF ACTION OF 2, 4-DICHLOROPHENOXYACETIC ACID

By P. L. GOLDACRE*

[Manuscript received April 12, 1949]

Summary

The rate of destruction of indole-3-acetic acid by a crude enzyme preparation from etiolated pea epicotyls is increased by 2, 4-dichlorophenoxyacetic acid.

A natural inhibitor present in boiled onion juice opposes this increase.

A mechanism is suggested for the action of 2, 4-D on the growth of plants.

I. INTRODUCTION

The use of synthetic plant growth-regulating compounds is of increasing economic importance in agriculture but little is known of their fundamental mechanism of action. The hormone-like character and general structural similarity of certain substituted aryl-acetates and -oxyacetates or their potential precursors (Zimmerman and Hitchcock 1941-42) to that of indole-3-acetic acid (I.A.A.) suggest that these compounds may act indirectly by altering the activity of this natural plant hormone. Tang and Bonner (1947) have partially characterized an enzyme prepared from pea epicotyls (and referred to here as I.A.A. oxidase) which oxidizes and inactivates I.A.A. On adding 11 auxin analogues to a crude enzyme preparation they found no change in the rate of I.A.A. inactivation. However, as these workers used a *substrate* (I.A.A.) *concentration* that was rate-limiting, the only rate-change necessarily to be detected would have been a *decrease* due to competition with the substrate.

II. EXPERIMENTAL AND RESULTS

In this laboratory a crude preparation containing I.A.A. oxidase (the "whole cytoplasm" of Tang and Bonner) was prepared from etiolated epicotyls of peas grown in the dark at 24°C. for seven to ten days. With an initial substrate concentration of 2×10^{-4} M the I.A.A.-destroying activity was found to be directly proportional to the enzyme concentration up to an activity of approximately 95 μ M/l./hr. at 27.5°C. This condition holds for subsequent batches of enzyme irrespective of the absolute activity per unit volume of the enzyme suspension, provided the initial substrate concentration and incubation temperature are the same. In experiments reported here the enzyme concentrations were always rate-limiting. On the addition of 2, 4-dichlorophenoxyacetic acid (2, 4-D), a

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marked stimulation in the rate of inactivation was obtained, increasing with increasing concentration of 2, 4-D (Fig. 1).

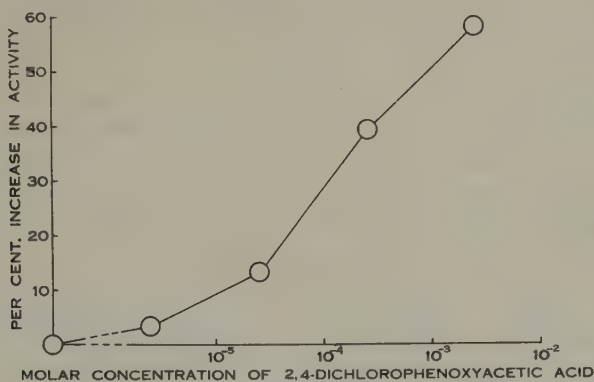


Fig. 1.—The effects of 2, 4-dichlorophenoxyacetic acid on the activity of a crude preparation of indole-3-acetic-acid oxidase. Each tube contains 0.5 ml. of 10^{-3} M indole-3-acetic acid, 0.5 ml. of M/15 phosphate buffer, pH = 6.64, 0.5 ml. of crude enzyme, the appropriate amount of 2, 4-D, and sufficient distilled water to make the total volume 2.5 ml. Incubated at 27.5°C. for 60 min.

Rates of inactivation of I.A.A. were measured by determining the residual concentration of I.A.A. at time intervals, using the $\text{FeCl}_3\text{-H}_2\text{SO}_4$ reagent of Tang and Bonner (1947). 2, 4-D alone or with boiled extract caused no destruction of I.A.A., nor did 2, 4-D interfere with the determination of I.A.A.

TABLE 1

EFFECT OF 2, 4-D AND BOILED ONION JUICE ON THE ACTIVITY OF CRUDE I.A.A. OXIDASE

Tube	Substance Added*		I.A.A. destroyed in 60 min. ($\mu\text{M/l.}$) †	Inhibition (%)
	10 ⁻² M 2, 4-D (ml.)	Boiled, Filtered Onion Juice (ml.)		
A	0	0	53.7	0
B	0.5	0	92.7	- 73
C	0	0.5	14.3	+ 73
D	0.5	0.5	31.3	+ 42

* Each tube contains 0.5 ml. of 10^{-3} M indole-3-acetic acid, 0.5 ml. of M/15 phosphate buffer, pH = 6.64, 0.5 ml. of crude enzyme preparation, and sufficient distilled water to make the total volume 2.5 ml. Incubated at 27.5°C. for 60 min.

† Arithmetic mean from three batches of crude enzyme. Statistical analysis made on square root transformed data which substantially equalizes the variance due to different relative activities of enzyme and inhibitor present in each batch. Effect of boiled extract on I.A.A. destruction significant at < 1 per cent. Effect of 2, 4-D on I.A.A. destruction significant at 6 per cent.

Tang and Bonner (1948) have reported that boiled extracts of several plants strongly inhibit the I.A.A. oxidase. In this laboratory it has been shown that such inhibition by boiled onion juice can be partly or wholly reversed by 2, 4-D. A set of data for a series of experiments is given in Table 1.

Tang and Bonner (1948) have been able to demonstrate the presence of a heat-stable inhibitor of I.A.A. oxidase in several plant tissues and in the crude enzyme preparation from such tissues. Since 2, 4-D can oppose the inhibition caused by added boiled plant extract, it is likely that the rate-stimulation observed on adding 2, 4-D alone to the crude pea enzyme is due to its reversal of the effect of the natural inhibitor present there.

It is here suggested that the enzyme *in situ* is normally functioning at suboptimal rates which are controlled by a heat-stable inhibitor present in the tissues. Applied 2, 4-D counteracts this inhibition, permitting a greater rate of I.A.A. destruction. As there appears to be a dynamic equilibrium between production of I.A.A. from tryptophane (Wildman, Ferri, and Bonner 1947) and its destruction by I.A.A. oxidase, the characteristic effects on plant growth produced by relatively large amounts of 2, 4-D might well arise through disturbing this equilibrium.

III. ACKNOWLEDGMENT

The work described in this paper was carried out as part of the research programme of the Division of Plant Industry, C.S.I.R.

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ON THE RELATIVE IMPORTANCE OF AEROBIC METABOLISM IN SMALL NEMATODE PARASITES OF THE ALIMENTARY TRACT

I. OXYGEN TENSIONS IN THE NORMAL ENVIRONMENT OF THE PARASITES

By W. P. ROGERS*

[Manuscript received February 28, 1949]

Summary

As a preliminary to the study of the relative importance of aerobic mechanisms in the metabolism of *Nippostrongylus muris*, *Nematodirus spathiger*, *Nematodirus filicollis*, and *Haemonchus contortus* at oxygen pressures of the normal environmental fluids of these parasites, the determination of oxygen in the contents of the small intestine of the rat and sheep, and the abomasum of the sheep, has been carried out. The method which was used allowed measurements to be made close to the mucosa of the alimentary canal of anaesthetized animals in which the circulation was left intact, and the procedure ensured that conditions in the normal gut were very little disturbed.

In the small intestine of the rat, oxygen tensions were found to vary from 30.2 to 8.9 mm. of mercury; these readings were usually lower as distances from the pylorus were increased. Oxygen was always present in the contents of the small intestine of the sheep close to the mucosa, but in smaller amounts than those found under similar conditions in the rat. As the oxygen tensions in regions very close to the intestinal mucosa of rats were influenced by the nature of the gases inspired by the animals, it is apparent that some oxygen diffused into the intestinal contents from the blood stream.

I. INTRODUCTION

A knowledge of the relative importance of aerobic and anaerobic mechanisms in the metabolism of nematode parasites of the alimentary canal is a necessary fundamental to most studies on the physiology of these animals. A direct experimental examination of the problem has not, as yet, been made *in vivo* nor, indeed, have satisfactory *in vitro* observations been made. The work of Slater (1925) and Davey (1938), who studied certain nematode parasites *in vitro*, must be considered inconclusive. Slater showed that starving *Ascaris lumbricoides*, when stimulated to activity under aerobic conditions, maintained movement longer than parasites in an oxygen-free medium, but it is doubtful if these results have much significance concerning the normal physiology of the actively feeding, largely motionless parasites *in vivo* (Archer and Peterson 1930). Davey found that several different trichostrongyle parasites of sheep, when placed in non-sterile media, survived longer under aerobic conditions. These results must be interpreted with caution because the growth of bacteria in the medium markedly influences the survival of nematode parasites *in vitro*, and the nature of the gas phase may have affected the parasites indirectly by influencing

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the nature and degree of bacterial growth. Davey, however, was aware of this complication and it is probable that bacterial growth, at least in his non-nutritive media, was slight.

The determination of the relative importance of aerobic and anaerobic metabolism in nematode parasites has been approached from another point of view by von Brand and Weise (1932) and Toryu (1934), who have shown that oxygen was either absent from the gut fluids of several host species, or present only in small amounts, thus indicating the unlikelihood of active aerobic mechanisms in parasites inhabiting those fluids. However, it might be expected on physico-chemical grounds that oxygen would diffuse from arterial blood, through the thin intestinal mucosa, into the lumen of the intestine; indeed, the permeability of the gut wall to gases has been demonstrated by McIver, Redfield, and Benedict (1926). It might be expected, then, that oxygen pressures, though very low in the bulk of the gut fluids, might be quite appreciable close to the gut mucosa. Methods needing relatively large samples of gut contents taken from dead animals might not indicate the true amounts of oxygen available to small parasites of the alimentary tract which usually live close to the mucosa or even between the villi (Porter 1935). It is clear then that the determination of the oxygen available to small nematode parasites *in vivo* should be carried out by the use of animals with an intact circulatory system, and the method should allow measurements to be made close to the mucosa of the alimentary tract without disturbing the milieu which exists in the normal animal.

The present work aims to define the limits of the oxygen pressures available to parasites in the alimentary tract of the rat and of the sheep. The ability of parasites to utilize oxygen at the pressures at which it occurs in their normal environments will be dealt with in a later publication.

II. PHYSICAL AND CHEMICAL METHODS

The electrometric method of Brink and Davies (1942) was considered suitable for the determination of dissolved oxygen in fluids of the alimentary tract. The use of this method allowed the measurements to be carried out in animals with the blood supply to the alimentary canal intact. Further, oxygen tensions in localized regions close to the intestinal and abomasal mucosa could be measured. Because results obtained with the "oxygen" electrode are sometimes variable (Brink and Davies 1942), the characteristics of the electrodes used in the present experiments will be described in detail to indicate the degree of accuracy which may be accorded to the results obtained.

(i) *The Electrodes*.—It was usually necessary to follow changes in oxygen pressures occurring over short periods of time, and open electrodes were therefore frequently used. However, the results were checked, whenever possible, by the use of recessed electrodes. The dimensions of typical electrodes were as follows:

Open electrode No. 2, outside diameter at tip, 0.96 mm., diameter of platinum at tip, 0.40 mm.

Recessed electrode No. 4, outside diameter at tip, 0.91 mm., diameter of platinum at tip, 0.65 mm., depth of recess, 0.56 mm.

The electrodes were mounted in 20-cm. glass holders; leakage to earth was reduced by coating the surface of the shaft of the holders with methylchlorosilane mixture.

The characteristics of the current-voltage and time-current relationships of open electrode No. 2 are shown in Figures 1 and 2. Open electrodes were

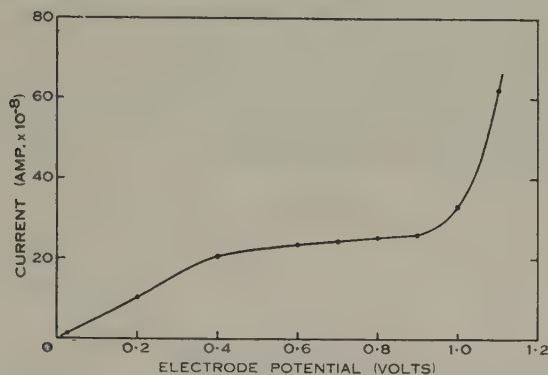


Fig. 1.—The current produced from open electrode No. 2 in air-saturated 0.15M NaCl at 24°C. at different potentials *v.* 0.15M calomel half-cell. Readings were taken 40 sec. after closing the switch.

calibrated before and after each set of experiments (Fig. 3). It was found that the change in the response of open-type electrodes over an experimental period of two hours might be as great as 20 per cent., but as a rule it was in the region of 10 per cent. Recessed electrodes were found to be more stable than open electrodes but not to the extent indicated by Brink and Davies (1942).

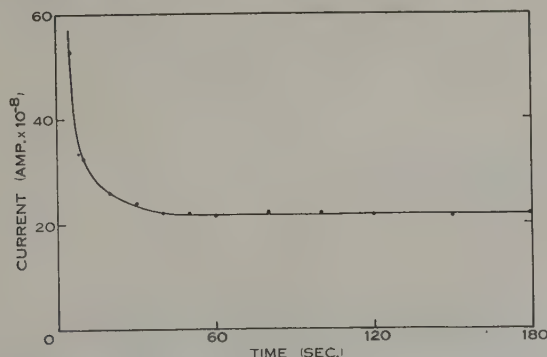


Fig. 2.—Time-current relationships of open electrode No. 2 in air-saturated 0.15M NaCl at 24°C. Electrode potential 0.8 volt *v.* 0.15M calomel half-cell.

When not in use, open electrodes were stored with tips in 0.9 per cent. saline; recessed electrodes were stored dry. Before each experiment the shafts

of the electrodes were wiped with a dry cloth. Before calibrating an electrode for a day's work, it was "run in" at a potential of 1.1 volts for 1 to 2 minutes. All these procedures increased the stability of the electrodes.

(ii) *The Measurement of Electrode Currents.*—Electrode currents were measured with a valve meter (Roberts 1939) calibrated from 5×10^{-5} to 1×10^{-9} amperes over 5 scales of 100 divisions. The electrodes were connected with the meter as shown in Figure 4. A 0.15M NaCl calomel half-cell was used to complete the circuit through a salt bridge. Electrodes and the half-cell were held in clamps arranged to give a high resistance to earth.

(iii) *The Calibration of the Electrodes.*—Physiological saline, warmed to 37°C ., in which the oxygen content was lowered by means of a stream of nitrogen, was used to calibrate the electrodes. Dissolved oxygen was estimated

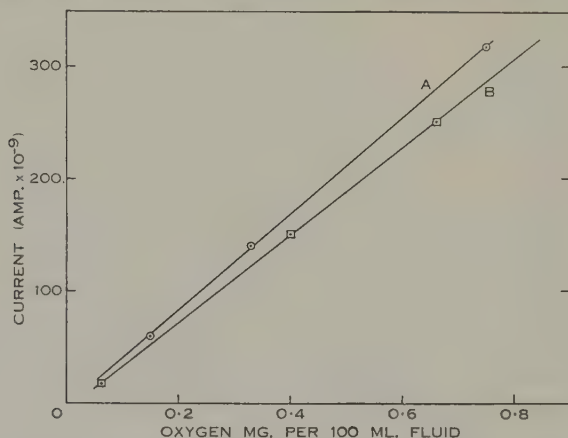


Fig. 3.—Current-oxygen tension curves of open electrode No. 2. Calibration was carried out using 0.15M NaCl at 37°C . containing known amounts of oxygen. Electrode potential 0.8 volt *v.* 0.15M calomel half-cell. Readings were taken 40 sec. after closing the switch.

chemically by Winkler's method, corrections being made for the addition of oxygen in the reagents (Krogh 1935). Calibration curves of open electrode No. 2 are shown in Figure 3.

III. BIOLOGICAL METHODS

Experiments with rats were carried out with animals of about 100 g. weight which had been starved for several hours before use. "Nembutal," 40 mg. per kg., given subcutaneously, was used as an anaesthetic. After being anaesthetized, the rats were tied to a small operating board equipped with clamps for the controlled positioning of electrodes.

The sheep were anaesthetized with "Kemithal" after atropine.

The surgical methods were simple and are described when reference is made to specific experiments.

IV. PROCEDURE AND RESULTS

The abdomen of an anaesthetized rat was shaved and a 2-cm. incision made in the body wall over the section of the small intestine to be examined. Bleeding of the cut surfaces was checked with 1/5000 adrenalin in saline. About 2 cm. of the small intestine was lifted gently through the incision and supported on warm saline-damped gauze. A small purse-string suture (000 silk) was placed in position on the intestine and a stab wound made in the centre of the suture. The intestine was gently pressed to expel a little of the intestinal contents, and any blood that was present, through the wound. The tip of an electrode which had just been calibrated was inserted in the stab wound, care being taken to avoid the introduction of air into the intestine. The suture was pulled tight around the shank of the electrode and the intestine was lowered into the body

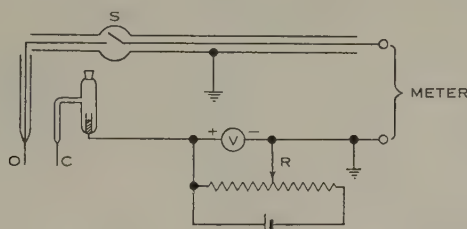


Fig. 4.—Method of connecting the electrodes to the meter. The switch, S, and the connection of the "oxygen" electrode, O, to the meter were covered by an earthed screen. The "oxygen" electrode and the calomel half-cell, C, were connected through a 0.15M NaCl bridge. A 300-ohm wire-wound potentiometer, R, and a 1½-volt dry cell were used.

cavity. The side of the electrode was then pressed gently against the intestinal mucosa, the calomel half-cell was lowered to make contact, through a fine capillary, with the surface of the intestine near the "oxygen" electrode, and the body cavity was closed with a loose suture. Ten minutes after inserting the electrode the meter was connected and the current readings were taken with the animal breathing either air, or 95 per cent. oxygen-5 per cent. carbon dioxide, or 95 per cent. nitrogen-5 per cent. carbon dioxide. At the end of the experiment the animal was killed with chloroform and the position of the electrode, in relation to the mucosa, and its distance from the pylorus were determined. If any blood was seen in the lumen of the intestine the result was discarded. Immediately the experiment was concluded, the "oxygen" electrode was recalibrated; if the current-dissolved oxygen calibration curves obtained before and after the experiment differed by more than 15 per cent., which was seldom, the results were considered too unreliable to use. In this manner, oxygen pressures in gut fluids near the mucosa of the small intestine were determined at different distances from the pylorus in a number of rats. Both normal rats and rats infected with *Nippostrongylus muris* were used.

The respiratory rate of the rats before they were anaesthetized was usually about 80-120 per minute. When oxygen pressures were being taken the rate was about 40-50 per minute, though it changed when the animals were allowed to breathe the different gas mixtures.

The results of these experiments are listed in Table 1. It can be seen that small amounts of oxygen were always present in the small intestinal contents of the rat close to the gut mucosa; the amounts found usually grew smaller as the distance from the pylorus was increased. On occasions, and especially when the electrode tip was pressed against the gut mucosa, the oxygen tensions recorded were markedly affected by the nature of the gas inspired by the experimental animals (see Fig. 5).

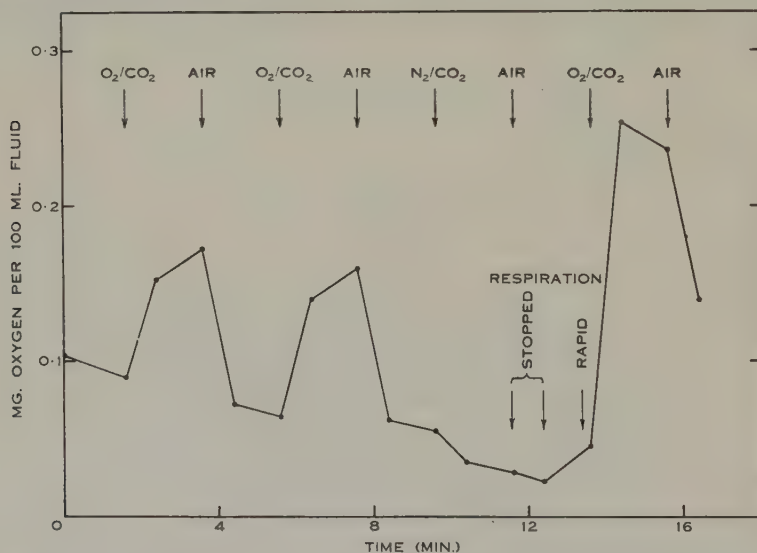


Fig. 5.—The effects of the nature of inspired gas mixtures on the oxygen content of the fluid very close to the mucosa of the rat small intestine. For further explanation, see text.

In the examination of the alimentary tract of the sheep, the cardiac end of the abomasum was first exposed and the electrode inserted into the fundus through a purse-string suture as before. After taking readings, the stab wound in the fundus was closed and a numbered tag fastened at the site. Readings were taken with the electrode inserted at the pyloric end of the abomasum and at several positions along the small intestine. Owing to the thickness of the wall of the abomasum, the position of the electrode tip in relation to the mucosa was difficult to assess. Also, it was difficult to avoid the introduction of air into the abomasal contents when placing the electrode in position. Other difficulties were caused by the long leads to the electrodes necessitated by the size of the animal. The results obtained from examination of the abomasum may thus have little significance. The effect on oxygen tensions in the gut fluids of allowing the animal to breathe nitrogen-carbon dioxide gas mixture was not examined because of the large number of determinations made on the one sheep.

At the conclusion of the experiment the animal was killed with chloroform, the alimentary tract removed, and the positions of the numbered tags marking the sites where determinations had been made were noted. Two animals were examined in this manner. The results obtained are given in Table 2. On two occasions when the "oxygen" electrode was pressed against the intestinal mucosa, a rhythmic rise and fall in the electrode current was noted. The readings indicated that the partial oxygen pressure rose as high as 17 mm. of mercury.

TABLE 1
OXYGEN TENSIONS IN THE CONTENTS OF THE SMALL INTESTINE OF THE RAT CLOSE TO THE MUCOSA AT DIFFERENT DISTANCES FROM THE PYLORUS

Position of Electrode: Distance from Pylorus (cm.)	Partial Pressure of Oxygen (mm. Hg at 16°C.)	Number of Parasites in Rat Intestine
2	23.5	None
5	14.3	None
6	21.8	None
12	30.2	Moderate
13	28.6	None
13	19.0	Moderate
15	23.5	None
19	19.0	Few
20	28.2	Few
25	22.2	Some solid food present
30	7.9	None
30	18.2	Moderate
35	17.5	None
43	19.0	None
75	11.2	None

During the experiments the respiratory rate of the sheep usually fell to between 8 and 10 per minute, less than half the rate noted before anaesthesia.

V. DISCUSSION

Apart from the inaccuracy of the actual method of measuring oxygen tensions as used in the present experiments, other factors influenced the reliability of the results obtained. Thus, although care was taken to avoid the introduction of air into the intestine when electrodes were inserted, the possibility that contamination with atmospheric oxygen gave rise to elevated oxygen tensions must be considered. However, ten minutes were allowed to pass after closing the intestine before the readings were taken. During this time, normal conditions might well have been established if contamination was slight. The observation that the pressure of oxygen recorded was sometimes influenced by the nature of the gas mixture inspired by the animal suggests that the oxygen reached the fluids of the gut by diffusion from tissues supplied with arterial blood. Hence it is probable that the lowered respiratory rates prevailing during experiments with anaesthetized animals led to abnormally low tensions in the alimentary

tract, for Campbell (1925) has shown that certain anaesthetics lower the pressure of oxygen in animal tissues. It would appear, then, that though some features of the present experiments may have led to increased oxygen tensions above the normal, other features, equally important, would reduce this error.

Heavy infestations with *Nippostrongylus muris* lead to a dilation of the blood vessels of the intestine where the parasites are localized in the region of 6 to 28 cm. from the pylorus. It is to be expected that the dilation of the blood vessels would cause an increase in the partial pressures of oxygen in the contents of the gut in this region. On the other hand, the utilization of oxygen by the parasites themselves, and the pathological enlargement of the lumen of the infected intestine, would reduce the oxygen tensions (Rogers 1948). The present experiments (see Table 1) did not reveal any difference between the infected and uninfected intestines.

TABLE 2
OXYGEN TENSIONS IN THE CONTENTS OF THE SMALL
INTESTINE AND ABOMASUM OF THE SHEEP CLOSE TO THE
MUCOSA AT DIFFERENT DISTANCES FROM THE PYLORUS

	Position of Electrode: Distance from Pylorus (cm.)	Oxygen Tension (mm. Hg)
Sheep 1	22	approx. 4.3
Abomasum	5	17.5
Sheep 1	52	12.7
Small intestine	114	4.7
	397	approx. 4.2
	804	approx. 4.0
Sheep 2	122	9.5
Small intestine	251	8.0
	336	approx. 4.1
	604	approx. 4.4

The results obtained in the examination of the small intestine of the sheep (see Table 2) were frequently two or three times as great as those obtained by von Brand and Weise (1932). This difference may well be due to the fact that, in the present experiments, the oxygen tensions relate to the thin layers of gut fluid adjacent to mucous membranes which retained an intact blood supply. The oxygen pressures found in fluids of the small intestine of the rat (see Table 1) were much higher than those found in that of the sheep. In the sheep the relatively smaller surface area per unit volume of the intestinal lumen would cause a more rapid fall in oxygen tension as distances from the mucosa towards the centre of the lumen of the intestine were increased. The intestines of the sheep used in these experiments contained considerable amounts of ingesta; the rat intestines, however, contained relatively little. These differences would again lead to lower oxygen tensions in the sheep intestine.

The tension of oxygen in the mucous membrane of the empty small intestine of the cat has been reported to be 35-40 mm. of mercury (McIver, Redfield, and Benedict 1926; Campbell 1932). It is not surprising, therefore, that oxygen tensions of the order indicated in Tables 1 and 2 were found close to the mucous membrane of the small intestine of the rat and sheep, even when ingesta were present. Further, oxygen tensions of 10-20 mm. of mercury (McIver, Redfield, and Benedict 1926; Campbell 1932) are found in the mucous membrane of the stomach of the cat, which again suggests that appreciable amounts of oxygen may be present in fluids close to the mucosa of the sheep abomasum. However, though there is little doubt that some oxygen was present at the sites examined in the present experiments, the amounts were low compared to those found in the usual aerobic environments. It remains to determine, therefore, whether the small nematode parasites found on the walls of the sheep abomasum or the rat and sheep small intestine are capable of utilizing oxygen when it is present in such small amounts. The function of haemoglobin in these parasites as a carrier of oxygen at low partial pressures of oxygen will be discussed in a later publication.

VI. ACKNOWLEDGMENTS

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ON THE RELATIVE IMPORTANCE OF AEROBIC METABOLISM IN SMALL NEMATODE PARASITES OF THE ALIMENTARY TRACT

II. THE UTILIZATION OF OXYGEN AT LOW PARTIAL PRESSURES BY SMALL NEMATODE PARASITES OF THE ALIMENTARY TRACT

By W. P. ROGERS*

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Summary

Nippostrongylus muris, *Haemonchus contortus*, *Nematodirus spathiger*, and *N. filicollis* are all capable of utilizing oxygen for respiratory purposes even when it is present at very low oxygen tensions. Thus with a partial pressure as low as 5 mm. of mercury the respiration of *Nippostrongylus muris* may reach 40 per cent. of its maximum rate, whereas *Nematodirus* spp. and *H. contortus* may respire at 25 and 12 per cent. of their maximum rates respectively. Further, the results indicate that *in vivo* the oxygen consumption rates may sometimes reach 80 per cent. of the maximum *in vitro* rate in the case of *Nippostrongylus muris* in the small intestine of the rat and 40 per cent. in the case of *Nematodirus* spp. in the small intestine of the sheep. *Haemonchus contortus* in the sheep abomasum probably respire at a relatively lower rate than either of the intestinal parasites *in vivo*.

Evidence which indicates that the maximum uptake of oxygen by the parasites *in vitro* may be much higher than *in vivo* has been presented. It is concluded that the oxygen tensions of the host gut fluids surrounding the parasites in their normal habitat may not greatly limit oxygen uptake *in vivo*, especially in the case of *Nippostrongylus muris*.

The oxygen consumption — oxygen tension curve for *Nippostrongylus muris* followed a hyperbolic course. When the results were treated according to the equation

$$A = \frac{P}{K_1 + K_2 P},$$

where A represents oxygen uptake, P is the oxygen pressure, and K_1 and K_2 are constants, P/A plotted against P gave a straight line with K_1 , the intercept, of 1.2, and K_2 , the slope, of 0.14. The sheep parasites gave similar results, with K_1 and K_2 values of 2.6 and 0.15 for *Nematodirus* spp., and 10 and 0.14 for *Haemonchus contortus*.

Mechanisms of oxygen transport used by the parasites are discussed.

I. INTRODUCTION

Previous work has shown (Rogers 1949) that oxygen is present in the contents of the alimentary tract of the rat and of the sheep in appreciable amounts when determinations are made close to the mucosa in animals which have an intact alimentary circulation. In the present paper the results of experiments designed to determine the ability of certain nematode parasites to utilize

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oxygen at the partial pressures found in their normal environments are discussed. The animals examined were *Nippostrongylus muris* from the rat small intestine, *Haemonchus contortus* from the sheep abomasum, and *Nematodirus flicollis* and *N. spathiger* from the sheep small intestine. These parasites were selected because it was thought that they might be small enough to allow oxygen to penetrate to their central tissues in the absence of a circulatory system, and because all these parasites contain haemoglobins which have very low loading tensions (Rogers, unpublished data). The functions of the haemoglobins of the parasites in the transport of oxygen will be discussed in a later publication.

Nematode parasites have been shown to utilize oxygen from saline solutions in equilibrium with air at 38°C. (Laser 1944; von Brand 1934; Rogers 1948). The animals used in the present investigation all consume oxygen at a relatively high rate (Rogers 1948; Lazarus, unpublished data). The oxygen consumption of larger parasites such as *Ascaris lumbricoides* or even *Ascaridia galli* increases when an oxygen atmosphere is used instead of air (Laser 1944; Rogers 1948). High oxygen tensions are, however, toxic to *Ascaris lumbricoides*; the low concentrations of catalase present in this animal's tissues apparently allow fatal concentrations of hydrogen peroxide to accumulate (Laser 1944). However, Laser states that the pattern of the oxidative enzyme system of *Ascaris* seems to show a perfect adaptation for functioning at low oxygen tensions. If even a minor part of the metabolism of the large *A. lumbricoides* in its natural habitat can be considered to be aerobic, the importance of aerobic mechanisms in small nematode parasites may be considerable.

II. METHODS

(i) *Biological Materials*.—*Nippostrongylus muris* was obtained from rats which had been infected experimentally. The rats were starved overnight, then killed, and the parasites were washed from the intestine with saline; the contaminating material was removed with a Pasteur pipette. The parasites were used within a few hours of the death of the host.

Nematodirus flicollis, *N. spathiger*, and *Haemonchus contortus* were obtained from naturally infested sheep. The parasites were removed from ingesta with light forceps. The cleaned worms were ready for use about four hours after the death of the host animals. The two species of *Nematodirus* were not separated for use.

(ii) *Manometric Methods*.—Oxygen uptakes were determined in saline at 38°C. in small Warburg flasks with K_{O_2} 's of the order of 0.3. With such vessels, using the "direct" method of Warburg (1926), adequate readings could be obtained with 50 mg. wet weight of material at oxygen partial pressures above 38 mm. of mercury. At oxygen pressures of 4 to 8 mm. of mercury, even 100 mg. of tissue, the largest amount which could be safely used in the vessels, did not give large enough readings to furnish accurate results. It is considered that measurements carried out manometrically at the lower pressures of oxygen may have errors as much as ± 15 per cent.

Gas mixtures were prepared with cylinder nitrogen and air over saturated sodium chloride solutions. The measurement of the volumes of the gases used in the mixtures was accurate to within ± 7 per cent. Each Warburg vessel was gassed with 2½ litres of gas mixture at room temperature. Q_{O_2} values (μ l. of gas taken up per hour per mg. dry weight of respiring material) were calculated from readings taken over the first 30 minutes.

At the end of each experiment *Nematodirus* spp. and *H. contortus* were taken from the Warburg vessels, dried on filter paper, and weighed. The small *Nippostrongylus muris* were counted. The dry matter content as related to wet weight or to the number of parasites was obtained on several occasions, giving factors that were subsequently used for converting results to a dry weight basis in the calculation of Q_{O_2} values.

TABLE I
THE EFFECT OF WARBURG SHAKING RATE ON THE X_{O_2} OF *NEMATODIRUS* SPP.
FOR PERIODS OF 15 MIN. AT AN OXYGEN PARTIAL PRESSURE OF 3.8 MM. OF
MERCURY AT 38°C.

Period	X_{O_2} (μ l./100 mg.) wet tissue)	Shaking Rate (7 cm. swing) per Minute
1	- 2.96	70
2	- 4.25	95
3	- 4.40	105
4	- 4.32	120
5	- 2.81	70

III. PROCEDURE AND RESULTS

Oxygen uptakes were determined manometrically at oxygen partial pressures of 3.8 to 152 mm. of mercury. In the lower range of oxygen tensions and with a V_F of 0.5 ml., the oxygen uptake was independent of the Warburg shaking rate above 105 per minute through a travel of 7 cm. (Table 1). When the speed of shaking was adequate at a given oxygen tension the oxygen uptake was directly proportional to the amount of respiring tissue within the limits of the amounts suitable for the particular Warburg vessels used (50 to 100 mg. wet weight). With oxygen partial pressures of 15 mm. of mercury and more, a shaking speed of 95 per minute was adequate.

The results varied somewhat with different lots of the same species of parasite. The variation, which was greatest at low partial pressures of oxygen, was probably partly due to the fact that the animals tended to get twisted into clumps in the Warburg vessels, especially when the units were shaken rapidly.

Results obtained with *Nippostrongylus muris*, *Haemonchus contortus*, and *Nematodirus* spp. are shown in Figures 1 and 2. The maximum pressures of oxygen found in the host fluids normally inhabited by *Nematodirus* spp. and *Nippostrongylus muris* are also shown. The oxygen tension of the contents of the sheep abomasum is not given, as the results reported previously (Rogers 1949) were taken from one animal only and so may possibly be misleading.

In *N. muris*, it would appear that even if the oxygen requirements of the parasite were as great *in vivo* as *in vitro*, enough oxygen would sometimes be

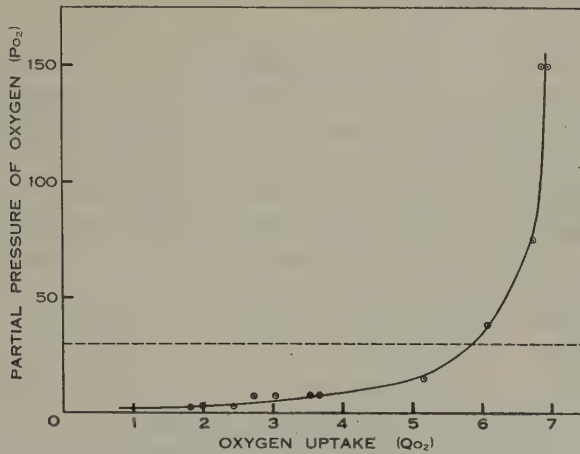


Fig. 1.—The relationship between the partial pressure of oxygen (mm. of mercury) and the oxygen consumption ($\mu\text{l.}/\text{hr.}/\text{mg. dry weight}$) of *Nippostrongylus muris*. The broken line indicates the upper level of the oxygen tensions found close to the mucosa in the small intestine of the rat.

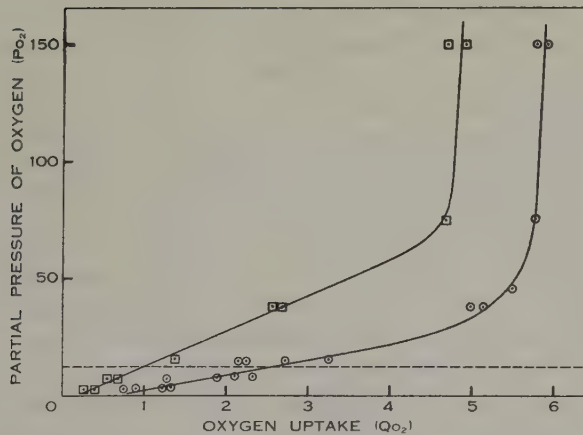


Fig. 2.—The relationship between the partial pressure of oxygen (mm. of mercury) and the oxygen consumption ($\mu\text{l.}/\text{hr.}/\text{mg. dry weight}$) of *Nematodirus* spp. (circles) and *Haemonchus contortus* (squares). The broken line indicates the upper level of the oxygen tension close to the mucosa in the small intestine of the sheep.

available in the normal environment to allow respiration to reach 80 per cent. of the maximum. *Nematodirus* spp. may be expected to have, on some occasions

at least, enough oxygen to allow 50 per cent. of the maximum respiratory activity. *H. contortus*, in the sheep abomasum, probably respire at a relatively lower rate than either of the intestinal parasites.

IV. DISCUSSION

In interpreting the results obtained in the present investigation, consideration must be given not only to the reliability of the determination of oxygen tensions in host gut-fluids (see Rogers 1948), but also to the rate of diffusion of oxygen through the fluids surrounding the parasites *in vivo*. Clearly, the rate of movement of gut contents over the surface supplying the oxygen, which may be taken to be the intestinal mucosa, will be small compared to the rate of mixing of the gas phase with the fluid phases in the Warburg vessels. However, it is probable that the nematode species examined in the present work live close to the mucosa of the host's alimentary tract, in which case even a slow rate of diffusion would not necessarily limit the oxygen available for the parasites.

The relative ability of the three species of nematode parasites to use oxygen at low partial pressures is clearly shown by comparing the oxygen uptake at a partial pressure of 5 mm. of mercury. In such an environment the respiration of *Nippostrongylus muris* would reach 40 per cent. of the maximum *in vitro* rate, and that of *Nematodirus* spp. and of *Haemonchus contortus* 25 and 12 per cent. respectively. The amount of oxygen found in host fluids (Rogers 1949) would indicate the oxygen consumption *in vivo* may sometimes reach 80 per cent. of the maximum rate *in vitro* in the case of *Nippostrongylus muris* and 40 per cent. in the case of *Nematodirus* spp. *Haemonchus contortus* in the sheep abomasum probably respire at a relatively lower rate than either of the intestinal parasites. It must be emphasized, however, that these figures relate to the maximum *in vitro* rate of oxygen consumption. Even at low oxygen tensions all the species of nematodes used in the present investigation made intensely active movements *in vitro* at 38°C. This activity was not apparent *in vivo*; at least, it was not evident in parasites in anaesthetized or freshly killed animals. Brody (1945) states that the ratio of sustained hard work to rest energy is probably "independent of size or species as such." In man or the horse, energy requirements for highly active movements as compared to basal metabolism energy may vary from 10 or even 20 to 1 (Dill 1936; Robinson, Edwards, and Dill 1937). If it is reasonable to apply these results to nematode parasites, it would appear that the maximum oxygen consumption of the highly active parasites *in vitro* may be as much as twenty times as great as the maximum consumption of the sluggishly motile parasites *in vivo*. It is quite possible, then, that conditions *in vivo*, especially for *Nippostrongylus muris* and *Nematodirus* spp., may allow oxygen consumption rates to approach the maximum *in vivo* rate.

A factor which has not been considered in this argument is that concerned with egg production by the parasites. Egg production falls very rapidly *in vitro*, though it may be quite high for short periods after the parasites are taken from the host. It is reasonable to suppose, then, that oxygen requirements for the energy of egg production by nematode parasites may be greater *in vivo* than

in vitro. The importance of this in assessing the relationship between maximum oxygen uptake *in vivo* and *in vitro* is difficult to determine. However, egg production may be classed, like other productive processes, as a growth process (Brody 1945) and so the oxygen requirements for the growth of *Tubifex tubifex* may give some indication of the order of the oxygen requirements for nematode egg production. Brody (1945, quoting Collier 1942) states that the oxygen uptake at the peak of regeneration in *Tubifex tubifex* rose 85 per cent. above the normal level. This suggests that the correction for the lowered egg output of the nematode parasite *in vitro* may not be very great and does not seriously invalidate the suggestion that oxygen may sometimes be available in such amounts *in vivo* as to allow small parasites of the alimentary tract to respire quite actively.

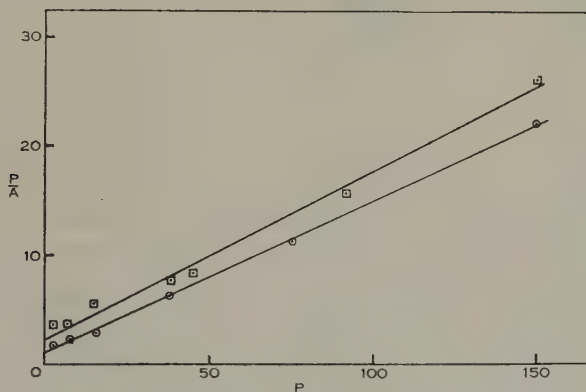


Fig. 3.—The relationship between P and P/A where P , the partial pressure of oxygen, is given in mm. of mercury, and A , the oxygen uptake of *Nippostrongylus muris* (circles) and *Nematodirus* spp. (squares) is given in $\mu\text{l./hr./mg. dry weight}$. The intercepts and slopes of the curves give the respective values of K_1 and K_2 for

$$\text{the equation } A = \frac{P}{K_1 + K_2 P}.$$

The results obtained in the study of the oxygen consumption of *Nippostrongylus muris* and *Nematodirus* spp., when treated according to the relation

$$A = \frac{P}{K_1 + K_2 P} \dots\dots\dots (1)$$

where A = oxygen uptake in ml. per hour per g. dry weight, P = oxygen pressure in mm. of mercury, and K_1 and K_2 are constants, show a linear relationship (Fig. 3) in which the intercept, K_1 , = 1.2 for *Nippostrongylus muris* and 2.6 for *Nematodirus* spp., and the slope, K_2 , = 0.14 for *Nippostrongylus muris*, and 0.15 for *Nematodirus* spp. The results obtained with *Haemonchus contortus* were more scattered than those for the other species, but indicated that K_1 was approximately 10 and K_2 about 0.14. These results are compared with some of those given by Tang (1933) in Table 2.

Equation (1) is similar to that developed by Langmuir (1918) to account for the absorption of gases on a solid surface and that describing the dissociation curve of oxyhaemoglobin (Barcroft 1928). Gerard (1931), assuming that oxygen was transported by an intermediary carrier or enzyme, also derived a similar type of equation for cell respiration. Working with yeast, Winzler (1941) found that at very low oxygen tensions the combination of oxygen with an oxygen-transferring enzyme was the process which determined the shape and constants of the curve relating oxygen uptake with oxygen pressure.

TABLE 2

THE VALUES OF K_1 AND K_2 FOR THE EQUATION $A = \frac{P}{K_1 + K_2 P}$ FOR SEVERAL DIFFERENT SPECIES (FOR FURTHER EXPLANATION, SEE TEXT)

Material Used	Temperature (°C.)	K_1	K_2
<i>Nippostrongylus muris</i>	38	1.2	0.14
<i>Nematodirus</i> spp.	38	2.6	0.15
<i>Haemonchus contortus</i>	38	10	0.14
<i>Planaria agilis</i>	20	137.0	7.4
Unfertilized eggs of			
<i>Arbacia punctulata</i>	25	6.0	2.10
Fertilized eggs of			
<i>Arbacia punctulata</i>	25	6.5	0.55
Fragments of			
<i>Chironomus thummi</i> larvae	16.5-23.0	42.5	2.02
Earthworm	25	28.0	5.25
<i>Termopsis nevadensis</i>	20	0.06	0.0043
Yeast	37	0.2	0.02

The relationship between the tension of oxygen on the surface of a nematode parasite and the oxygen tensions at the central tissues of the parasite resulting from diffusion processes may be approximately determined by a treatment similar to that devised by Fenn (1928) for the study of the penetration of oxygen into nerves. The parasites may be considered to be cylinders of tissue of radius a consuming A ml. of oxygen per hour per g. dry weight, at an oxygen pressure on the surface of P_0 cm. of mercury. The diffusion constant, D , may be taken as that assessed for muscle by Krogh (1919), 8.4×10^{-4} ml. of oxygen diffusing across a surface area of 1 cm.² per hour under a pressure of 76 cm. of mercury. In any concentric cylindrical layer of tissue of radii r and $r+dr$ within the parasite, the oxygen consumption in time, dt , is equal to the amount of oxygen diffusing into the layer minus the amount leaving the layer in the same time. Under these circumstances an equation

$$rDd^2c/dr^2 + Ddc/dr = Ar \dots \dots \dots (2)$$

can be derived if the passage of oxygen in and out of the ends of the cylinder is neglected. This equation is similar to that obtained by Fenn (1928) except for the additional factor r in the first term. A solution of the differential equation (2) is

$$P = P_0 - A/4D(a^2 - r^2) \dots \dots \dots (3)$$

where P is the oxygen tension in cm. of mercury at a distance r cm. from the

centre of the cylinder. If $r = 0$, P gives the oxygen tension at the centre of the parasite of radius a . By using appropriate values of P_0 , A , D , and a , the oxygen concentrations in the central tissues of *Nippostrongylus muris*, *Nematodirus* spp., and *Haemonchus contortus* can be calculated. Table 3 lists the radii of the nematodes examined and the oxygen pressures in the medium necessary to give zero oxygen tensions in the central tissues. The radii given are average values obtained by measuring ten mixed males and females of each species at

TABLE 3

OXYGEN TENSIONS AT THE SURFACES OF NEMATODE PARASITES OF DIFFERENT SIZES NECESSARY TO GIVE ZERO OXYGEN TENSIONS IN THE CENTRAL TISSUES

Species of Parasite	Radius of Parasite (mm.)	Surface Oxygen Tension Giving Zero Tensions at the Centre (mm. of Hg)
<i>Nippostrongylus muris</i>	0.051	16
<i>Nematodirus</i> spp.	0.077	21
<i>Haemonchus contortus</i>	0.14	32

five points along their length. The results obtained by means of equation (3) are merely first approximations, for the equation makes no allowance for variations in A and D in different tissues of the parasites. However, the method is sufficient to show that at low partial pressures of oxygen the fall in oxygen consumption by the parasites is much slower than would be expected from the rate, according to the equation, at which the anaerobic region in the central tissues increases. Thus, without some other form of physical or chemical oxygen-transporting system, diffusion would not be sufficiently rapid to allow the parasites to respire actively at low partial pressures of oxygen. There may be several different oxygen-transporting systems assisting the diffusion of oxygen in the parasites. *In vitro*, the parasites' alimentary tracts make sluggish movements, which, in disturbing the perienteric fluid, would assist oxygen transport. Further, the presence of haemoglobins of low loading tensions and cytochrome in the parasites (Rogers, unpublished data) suggests that oxygen transport other than diffusion is of importance in the species of nematodes which have been examined.

Though nematode parasites do not appear to take up fluids *per os* under the conditions used in the present experiments *in vitro*, those species which have been examined *in vivo* appear to feed very rapidly (Wells 1931; Nishi 1933; Rogers and Lazarus 1949a). It would appear, then, that the ingestion of host tissues or fluids from the surface of the host gut mucosa might provide oxygen which could be absorbed through the alimentary tracts of the parasites. Calculation shows that this source of oxygen would be inadequate in the case of *Ascaridia galli*. However, in smaller parasites the result might be quite different, especially if the rate of passage of materials along the parasite alimentary tract was comparable to that of *Ancylostoma* (Wells 1933). In the absence of precise knowledge concerning the feeding habits of the animals used in the

present investigation no definite statement can be made concerning alimentary oxygenation. In *Nippostrongylus muris*, which feeds on host tissue and blood (Rogers and Lazarus 1949a; Rogers, unpublished data), it is probable that some oxygen is delivered to the central tissues via its alimentary tract.

Consideration of the results obtained in the present investigation leads to the suggestion that aerobic mechanisms of certain *trichostrongyle* parasites may be important in the economy of these animals. As yet, studies on the intermediary metabolism of these organisms have been confined to anaerobic processes of energy production arising from the breakdown of glycogen to pyruvate and lactate (Rogers and Lazarus 1949b). It is quite probable that such processes are preliminary to the more efficient oxidative processes of energy production such as the tricarboxylic acid cycle. The nature of such processes in nematode parasites is being examined.

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A STUDY OF THE PROCESSES OF DIGESTION IN CERTAIN INSECTS

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Summary

Several aspects of the processes of digestion in the insects *Blattella germanica*, *Periplaneta americana*, and *Tenebrio molitor* are reported. In *Blattella* starved for 2 days, a meal of coloured starch reaches the midgut within 10 minutes and the rectum within 5 hours. The pH of the gut contents on a starch diet is approximately 4.5 in the crop, 6.0 in the midgut, and 8.0 in the hindgut. A protein diet raises the pH of the crop to about 6.0, but does not change that of the other regions. There is a gradient of decreasing redox potential from the crop to the hindgut where the *Eh* approximates -0.1 V. at pH 8.0.

Concurrent quantitative enzyme estimations and cytological investigations on *Blattella* have proved that the presence of cytoplasmic globules, hitherto generally referred to as cytological evidence of secretory activity, is not associated with an increase in enzyme concentration in the gut contents. The greatest enzyme concentrations are found when the cytoplasm is cytologically uniform. The secretory globules are more probably signs of cell breakdown than an indication of secretory activity.

Digestive enzymes are still present in *Blattella* midgut contents after 3 days' starvation, but the enzymes studied increase in concentration when the insect is fed, irrespective of the diet. A digestive enzyme of *Blattella* decreases in amount when the insect is fed a diet of that particular enzyme substrate for some time. The enzyme concentration is fairly slow to regain its former level.

Evidence is presented that stimulation of epithelial regeneration of the midgut of *Tenebrio* is effected by a factor carried in the blood. There is some evidence, mainly morphological, against the nervous control of midgut secretion.

A study of the localization of various substances shows that different materials may be absorbed in different regions of the gut. Fore-, mid-, and hindguts, and the midgut caeca may all be involved in absorption. The histopathology of a number of insecticides suggests that, except for arsenic compounds, changes produced in the midgut are not sufficient to account for death of the insect.

I. INTRODUCTION

In spite of a considerable number of investigations, there are many problems concerning the digestive processes of insects on which we have inadequate information. This is not surprising, since the same has quite recently been said of vertebrates (Babkin 1944), but the whole process in insects is probably simpler, and a knowledge of it is a necessity for the intelligent formulation of certain types of control measures. We have applied a number of experimental techniques to the study of the alimentary canal, particularly of the German cockroach *Blattella germanica* (L.), although other species have been used when *Blattella* was unsuitable.

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Many authors have studied the cytology of intestinal secretion in insects, but there is still considerable doubt about the nature of cell inclusions and their relation to enzyme formation; perfunctory examination of the relation of enzyme production to diet has not yet proved whether digestive enzymes are produced in response to secretagogues or other stimuli or whether they are all produced continuously; while information has been obtained on the site of absorption of some inorganic ions, practically nothing is known of the absorption of organic materials; and the whole problem of intermediary metabolism is almost unexamined. To attempt to elucidate these and related problems the following investigations were undertaken. Details of the methods employed will be found in each section.

The culture of *Blattella* was maintained at a fairly constant temperature of 32°C. and a relative humidity of about 70 per cent. Abundant supplies of cut potato, bran mash, and water were available at all times in the stock cultures. The individuals used in experiments were fed an artificial diet composed of ground whole wheat, dried milk powder, dry yeast, sugar, and fat. Thriving cultures of the larger cockroach, *Periplaneta americana* (L.), and of the mealworm, *Tenebrio molitor* L., the other species used, were maintained at about 27°C. and about 35 per cent. relative humidity.

II. MORPHOLOGY

The mouthparts, feeding mechanism, and gross morphology of the gut of *Blattella* have been well described by Snodgrass (1944, Figs. 7 and 8) and the morphology and histology of the gut have been studied by Ross (1930). The proventriculus has been described in considerable detail by Judd (1948). Two aspects of the morphology of the *Blattella* gut, which are important from the physiological viewpoint but which have not received attention, are the tracheation and innervation. The tracheal supply of the gut is of Snodgrass's generalized type C (1935, Fig. 223, p. 430), in which visceral tracheae are given off from each abdominal spiracle. In addition to the possibility of ventilation through the lateral trunks, conspicuous anastomoses between relatively large tracheae are found on the haemocoelic surface of the midgut, but not in other regions. The dark field photomicrographs (Plate 5, Figs. 28 and 29) illustrate differences in the form of tracheolar endings in different regions of the gut. In the crop (Plate 5, Fig. 28) fine tracheal trunks send short branches through the muscularis to penetrate the epithelium. Silver nitrate preparations, as used to demonstrate ascorbic acid, show that the tracheoles penetrate some of the epithelial cells. In the midgut (Plate 5, Fig. 29) relatively large tracheae send very short branches with conspicuous end-twigging to and through the muscularis so that every epithelial cell is supplied by tracheoles. This organ is more thoroughly tracheated than any other region of the alimentary tract. In the hindgut the epithelium is composed of patches of cuboidal cells with flattened epithelial cells between the patches. The tracheae run to these patches, where they branch repeatedly to form an intertwining mass of tracheoles. The low epithelial cells are less well tracheated.

The innervation of the gut as seen in methylene blue preparations is described in Section VII below.

III. TIME REQUIRED FOR PASSAGE OF FOOD THROUGH THE ALIMENTARY TRACT

Snipes and Tauber (1937) and Snipes (1938) have studied the rate of passage of food through the gut of *Periplaneta*, and the effect of various poisons on it. They were mainly concerned with egestion time, that is, the period from ingestion to the passage of faecal pellets, but Snipes gives some data on the time taken for banana paste to reach various points in the gut. We have obtained similar data for adult *Blattella* starved for 2 days and then fed on starch paste coloured with carmine, trypan blue, or orange G. Typical results are illustrated in Figure 1, in which the position in the gut reached by food after various time intervals is shown by horizontal lines. Broken lines indicate

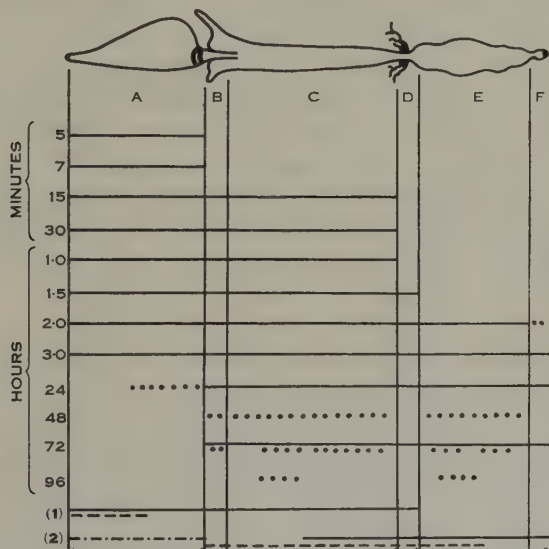


Fig. 1.—Diagram illustrating the passage of food through the *Blattella* alimentary tract. See text for description. A, crop; B, caeca; C, midgut; D, ileum; E, large intestine; F, rectum.

the presence of traces only of colouring matter from the food. Food is held for a short period in the crop by the proventriculus. The next delay is at the entrance of the malpighian tubules into the gut. Passage through the midgut and through the hindgut, once it begins, is relatively rapid. The two examples at the bottom of the figure illustrate the effect of feeding starch paste of one colour and replacing it by another. Thus in (1) after the insect had fed for 20 minutes on carmine paste, it was transferred to trypan blue paste, and examined 20 minutes later. The movement of the blue colour only part way into the crop after 20 minutes indicates the effect of the state of nutrition on the rate of passage. In (2) an adult *Blattella* was fed for 20 minutes on carmine starch

(solid line), then transferred for 2½ hours to trypan blue starch (broken line), and then transferred for 1 hour to orange G starch, after which the midgut was removed and examined. It will be observed that there was some mixing of the first two meals in the hind part of the midgut and the anterior part of the large intestine, but that all the middle meal (trypan blue starch) had passed through the crop into the midgut, its place being taken by the third meal.

The conclusions from these experiments are : (1) In an individual starved for two days, the crop is filled and some food has reached the midgut within 5 minutes. (2) The midgut is filled within 20 minutes, but there is no sign of food in the hindgut until about 2 hours after feeding. (3) The hindgut is slowly filled until after 4 hours it contains material as far as the rectum. (4) If a meal is fed to a replete insect, the second meal has penetrated only as far as the crop after 20 minutes. (5) Some mixing of successive meals can occur in the mid- and hindguts. (6) After a full meal at least three days are required for the crop to empty again. In some insects some dye still remained in granules in the mid- and hindguts 96 hours or even longer after the insects had been removed from food. A readily metabolized material would, of course, have all disappeared from the gut by this time, and insects subsequently fed a normal diet appeared to contain less of the dye than those starved.

From these observations it is clear that: (1) we may consider *Blattella* to be starved when it is removed from all food for 3 days; and (2) on feeding, the cells of the midgut will have an opportunity to react to the presence of food within 10 minutes.

IV. HYDROGEN ION CONCENTRATION AND OXIDATION-REDUCTION POTENTIAL

The pH of the contents of the alimentary canal of insects has been quite extensively studied (see Waterhouse (1940) for review). Wigglesworth (1928) employed a colorimetric comparator method for the study of the *Blattella* gut and found that the pH varied in different regions from a minimum of 4.6 in the crop to a maximum of 6.4 in the midgut but he did not study the pH of the hindgut. He found that the pH of the crop contents was higher after a protein diet than after a carbohydrate one.

Waterhouse (loc. cit.) discussed the methods that are available for the study of the pH of gut contents and concluded that the most satisfactory is to include a series of indicators in the food. We have employed this method in a study of *Blattella* and have extended Wigglesworth's observations in several respects. Cockroaches are not as satisfactory for this investigation as the Diptera which Waterhouse studied, since the contents of the mid- and hindguts are normally brownish and this colour tends to interfere with delicate changes in the colours of indicators. We have succeeded in overcoming this difficulty when necessary by puncturing the gut and comparing the colour of the contents with the colour of the unpunctured gut. Even though the observations cannot be made as accurately as those of Waterhouse, changes in pH are apparent in short sections of the gut, for example, in the vicinity of the malpighian tubules, which would never show up in the method employed by Wigglesworth since it

invariably involves some mixing of the gut contents. The sulphonphthalein indicators were used because of their relatively low salt and protein errors.

Tables 1 and 2 show the results obtained. In general the crop contents are somewhat acid, those of the caeca and midgut almost neutral, while those of the hindgut and in the region of the malpighian tubules are alkaline. A carbohydrate diet reduces the pH of the crop contents, but does not change those of the midgut or hindgut.

TABLE 1
pH OF GUT CONTENTS OF *BLATTELLA* FED STARCH + INDICATORS

Indicator	Crop	Caeca	Midgut	Ileum	Hindgut
Thymol blue	> 2.6 < 8.0	> 2.6 < 8.0	> 2.6 < 8.0	> 2.6 < 8.0	> 2.6 < 8.0
Brom-phenol blue	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5
Brom-cresol green	c. 4.6	> 5.2	> 5.2	> 5.2	> 5.2
Brom-thymol blue	< 6.5	< 6.5	< 6.5	> 7.2	> 7.2
Phenol red	< 7.0	< 7.0	< 7.0	< 8.0	< 8.0
Cresol red	< 7.4	< 7.4	c. 8.4*		< 7.4*
Range	> 4.5 < 6.5	> 5.2 < 6.5	> 5.2 < 6.5	c. 8.0	c. 8.0

* Anomalous results.

TABLE 2
pH OF GUT CONTENTS OF *BLATTELLA* FED GELATINE + INDICATORS

Indicator	Crop	Caeca	Midgut	Ileum	Hindgut
Thymol blue	> 2.6 < 8.0	> 2.6 < 8.0	> 2.6 < 8.0	> 2.6 < 8.0	> 2.6 < 8.0
Brom-phenol blue	> 4.5	> 4.5	> 4.5	> 4.5	> 4.5
Brom-cresol green	> 5.2	> 5.2	> 5.2	> 5.2	> 5.2
Brom-thymol blue	< 6.5	< 6.5	< 6.5	> 7.2	> 7.2
Phenol red	< 7.0	< 7.0	< 7.0	> 8.0	> 8.0
Cresol red	< 7.4	< 7.4	< 7.4	c. 8.4	< 7.4*
Range	> 5.2 < 6.5	> 5.2 < 6.5	> 5.2 < 6.5	c. 8.0	c. 8.0

* Anomalous result.

An attempt was made to incorporate the indicators in the artificial diet but its brown colour interfered with the indicator colours.

The figures for the midgut agree closely with those of Wigglesworth, who has discussed them in relation to the pH optima of the digestive enzymes. In some individuals a small region immediately anterior to the point of entry of the malpighian tubules had the pH of the hindgut rather than that of the midgut. When this was noted the peritrophic membrane was coiled in this region.

It is noteworthy that the indicators always penetrated to the ends of the caeca. But they were never found in the haemocoel or in any organs other than the gut. If, however, the same dyes were injected into the body cavity many of them made their way into the alimentary canal.

The oxidation-reduction potential of the insect alimentary tract has not been extensively studied, although Linderström-Lang and Duspiva (1936) have obtained interesting data on the larva of the clothes moth, *Tineola*, indicating very low values in the midgut. We have incorporated redox indicators at a

concentration of 0.1 per cent. in starch fed to *Blattella*. At this concentration the indicators used would probably not alter the poisoning of the system. Consistent colour changes have demonstrated a decrease in redox potential from the fore to the hind end of the gut; that is, the increase in alkalinity of the gut contents is accompanied by a decrease in redox potential (Table 3). In the regions of low *Eh* the indicators regained their colour on exposure to air. While these data are only approximate, they are interesting in view of the scanty information on the redox systems in the insect gut.

TABLE 3
APPROXIMATE REDOX POTENTIAL (IN VOLTS) OF THE GUT OF *BLATTELLA* FED STARCH + INDICATORS

Indicator		Crop pH 4.5	Midgut pH 6.0	Hindgut pH 8.0
Indigo disulphonate	Colour	Blue	Green	Green
	<i>Eo</i>	$> + 0.02$	<i>c.</i> $- 0.07$	<i>c.</i> $- 0.15$
Indigo trisulphonate	Colour	Blue	Blue	Blue
	<i>Eo</i>	$> + 0.06$	$> - 0.03$	$> - 0.12$
Indigo tetrasulphonate	Colour	Blue	Blue	Colourless
	<i>Eo</i>	$> + 0.10$	> 0.01	$< - 0.09$
Methylene blue	Colour	Blue	Colourless	Colourless
	<i>Eo</i>	$> + 0.13$	$< + 0.03$	$< - 0.02$
1-Naphthol-2-sodium sulphonate-indo-2, 6-dibromo-phenol	Colour	Colourless	Colourless	Colourless
	<i>Eo</i>	—	$< + 0.15$	$< + 0.05$
Phenol-indo-2, 6-dibromo-phenol	Colour	Colourless	Colourless	Colourless
	<i>Eo</i>	—	—	$< + 0.12$
Range		$> + 0.13$	$> + 0.01 < + 0.03$	$> - 0.12 < - 0.09$

Some exceptions to the figures given in Table 3 were noted. Thus, methylene blue and potassium indigo tetrasulphonate were observed to be reoxidized in the rectum, indicating a much higher *Eh* in this region than in the hindgut. In one individual fed Janus Green, the indicator was present in the reduced condition in the posterior half of the midgut and in the hindgut, suggesting a much lower *Eh* for these regions than shown by the other indicators.

V. CYTOLOGY OF THE MIDGUT EPITHELIUM

(a) Normal Histology

In *Blattella* it is apparent, for reasons given in Section VI, that the midgut (with its caeca) is the principal organ concerned with the secretion of digestive enzymes. The midgut is also responsible for some absorption, although the large intestine and the crop also absorb certain substances. The midgut epithelium is, therefore, a tissue of considerable interest and has been extensively studied in insects. Petrunkewitch (1900) and Ross (1930) both figured the

histology of the midgut of *Blattella*, but considerable detail can now be added to their descriptions.

The insect midgut is always composed of a simple epithelium. In some large species it may be folded or convoluted to increase the surface area, but in *Blattella* there is a simple columnar and fairly uniform epithelium. There is, however, a gradual change in the shape of the cells from the anterior to the posterior end. At the anterior end the cells are tall and the nuclei are laterally compressed, while at the posterior end the cells are more cuboidal and the nuclei are almost spherical. Evenly distributed among the epithelial cells are groups of undifferentiated cells, the regenerative nidi (Plate 1, Fig. 2). Mitoses are frequent in these nidi, the cells of which replace the mature epithelial cells as the latter degenerate. There is a conspicuous striated border on the distal end of the cells and this also is shorter in the more posterior cells (compare Plate 1, Fig. 4, with Plate 1, Fig. 1), although it changes in length, perhaps during digestion. The cytoplasm in the anterior cells is denser distally, while in the posterior cells it is more uniform. In the cytoplasm, either basophilic or acidophilic granules are sometimes found, although in the active midgut they are not abundant. Some of these granules give a positive test for acid phosphatase by Gomori's (1941) technique, and some contain glycogen. Very occasionally a cell, always distant from a regenerative nidus, may be seen degenerating (see Plate 2, Fig. 8). The epithelium appears to be homomorphous following the usual histological techniques, but a distinct cell type, confined to the anterior end of the midgut, and containing conspicuous argentophil inclusions, is readily distinguishable by Bodian's silver technique, following alcoholic Bouin's fixative. Figure 5 shows that these inclusions are scattered throughout the epithelium, but that they occur in greater numbers distal to the nucleus. The junction between the region of argentophil cells and the cells lacking these inclusions (shown in Plate 1, Fig. 6) is very sharp. Following fixatives containing osmic acid the epithelium appears to contain two cell types (Plate 5, Fig. 30), since the oldest cells (see below) always stain darker. This is especially true after feeding on starch. In a number of cells the nucleus is indented at its proximal pole (Plate 1, Fig. 6) but this is not evident in Fleming-fixed material, suggesting that the indentation may represent a fixation artefact. There is usually a single nucleolus.

An estimate of the number of cells in the midgut epithelium can be made by two methods, either by dividing the average area of a cell (approximately 10 by 10 microns) into the area of the midgut ($\text{length} \times 2\pi r = 10 \times 2 \times 22/7 \times 0.5 \text{ mm.} = 31.4 \text{ sq. mm. approx.}$), or alternatively, by counting the number of cells in a complete longitudinal section (approximately 1000) and multiplying by the number in a 10 micron transverse section (approximately 300). Both methods give a figure in the vicinity of 300,000 cells in the *Blattella* midgut. The number of nidi in the midgut can similarly be estimated to approximate 40,000. No estimate has been made of the number in the caeca.

The epithelium is overlain by a thin connective tissue layer which binds the epithelium to the muscularis. It is usually inconspicuous but is well differen-

tiated by toluidine blue. The epithelial cells separate when placed in an extract of hyaluronidase prepared from hog testes, suggesting that they are bound together by substances similar to those of vertebrates. The muscularis consists of an inner layer of circular muscles and outer longitudinal muscles, which are together responsible for peristalsis. The midgut does not undergo continuous writhing movements as does the crop, but reacts to mechanical stimulus with a slow contracture. The flattened nuclei of the muscle cells may be clearly seen in Plate 1, Figure 6.

(b) *Effects of Starvation and Poisons*

Degenerating cells are occasionally observed in the epithelium of normal insects. When midguts of a series of *Blattella* starved for 1 to 5 days are studied, degenerating cells become more abundant with increasing periods of starvation. This degeneration may take the form of the extrusion of many droplets expressed through the striated border (Plate 2, Fig. 7) or of the extrusion of a nucleus and its adherent cytoplasm (Plate 2, Fig. 8). The majority of authors in the past have considered these droplets to represent "merocrine secretion." In a large series of *Blattella* we have both histological preparations and estimations of the concentration of the digestive enzymes, proteinase and amylase. From these data it is clear that the extrusions of cytoplasm are in no way connected with secretion, and they prove, in fact, that the highest enzyme concentrations are associated with epithelia exhibiting a very uniform cytoplasm (as in Plate 2, Fig. 9), hitherto generally referred to as a "resting epithelium." In general, the longer the insect is starved, the more frequent are the extrusions of cytoplasmic fragments, and the lower is the concentration of digestive enzymes.

TABLE 4
RESULTS OF INJECTION OF SODIUM ARSENITE INTO ADULT *PERIPLANETA*

		Controls	One Hour after Injection	Two Hours after Injection
Mitoses per 25 nidi: means of 10 counts		8.2	6.2	5.8
Proteinase (optical density): means of six individuals	Caeca	0.157	0.150	0.174
	Midgut	0.203	0.198	0.240

We have studied this matter further by investigating the so-called "hypersecretion" produced by poisons. Thus Hoskins (1940, p. 355) has reported data obtained by Wilson on "hypersecretion" in the midgut of *Pieris rapae* following injection or ingestion of sodium arsenite. Twelve normal *Periplaneta*, each approximately 1 g. in weight, were injected through the coxo-femoral joint with 1 ml. of 0.1M sodium arsenite. At the end of one hour all roaches showed signs of poisoning and after 2 hours several were moribund (compare Yeager and Munson 1945). It was shown that sodium arsenite in the concentrations employed had no effect on the quantitative estimation of proteinase by the colorimetric method referred to in Section VI below. Counts of mitoses, by the method given in Section VII, and proteinase estimations on the entire midgut and contents

were performed on the same individuals, while histological observations were made on midguts and caeca of insects treated simultaneously in the same way. The histological examination showed the typical "hypersecretion" (Plate 3, Fig. 17). The results of enzyme estimations and mitotic counts are given in Table 4.

None of these differences are significant, but because of the possibility that two hours would not be sufficient time to produce maximum cell breakdown, the experiment was repeated, permitting the poison to act for 2 and 4 hours after injection. In this series, only the peritrophic membrane and its contents were taken for the determinations of proteinase activity. The results are given in Table 5. The differences between treatments are again not significant, indicating

TABLE 5
PROTEINASE ESTIMATIONS IN *PERIPLANETA* POISONED WITH SODIUM ARSENITE

Controls		Two Hours after Injection		Four Hours after Injection	
Weight of Peritrophic Membrane and Contents (g.)	Proteinase (optical density)	Weight (g.)	Proteinase (optical density)	Weight (g.)	Proteinase (optical density)
0.0099	0.162	0.0065	0.184	0.0351	0.254
0.0093	0.201	0.0087	0.212	0.0120	0.070
0.0051	0.157	0.0060	0.119	0.0117	0.088
0.0152	0.123	0.0215	0.219	0.0046	0.311
0.0215	0.254	0.0203	0.349	0.0176	0.211
0.0167	0.327	0.0178	0.327	0.0081	0.116
Mean	0.204		0.235		0.176

that the striking cytological picture of globule formation (hypersecretion) is not accompanied by a comparable rise in either intra- or extracellular proteinase. The results given in Tables 4 and 5 suggest that there may be a slight increase in proteinase 2 hours after injection, and since the cytoplasm of the epithelium must contain proteinases, such an increase would indeed be expected. But it is clear that the tremendous cytological differences are associated with at most only a slight rise in digestive enzyme concentration. Since large amounts of proteinase are found without any visible cytological evidence it is clear that cytoplasmic globule formation is not the normal method of secretion by the *Blattella* or *Periplaneta* midgut epithelium.

A comparison of the data contained in Plate 3, Figures 16 and 17, and Tables 4 and 5 will reveal (i) that there is a more marked breakdown in the epithelium of both caeca and midgut following arsenite poisoning after 2 hours than after 1 hour, though the incipient changes are already clear at the end of 1 hour; (ii) the cells which show the first signs of breakdown are the oldest cells, i.e. the furthest from the regenerative nidi; (iii) the cellular breakdown follows the pattern of the breakdown which may rarely be seen in the normal epithelium, but

which becomes more marked following starvation; (iv) there is no destruction of the peritrophic membrane, as Wilson (see Hoskins 1940) described in *Vanessa*; (v) the number of mitoses does not increase as the epithelium breaks down following arsenite poisoning, but is progressively slightly reduced; and (vi) the proteinase concentration does not increase to the extent expected with increasing cell breakdown if cytoplasmic globule formation represented true secretion.

From these experiments it may be concluded that so-called "secretion droplets" do not indicate the normal mechanism of digestive enzyme secretion, and reports of "hypersecretion" must be accepted with caution unless accompanied by confirmatory quantitative enzyme determinations.

(c) *Effects of Feeding*

The effects on the epithelium of feeding are seen in Plate 2, Figures 9 and 10. Whatever the diet, the cytoplasmic fragments described above are no longer visible. Instead, the epithelium presents an appearance which has hitherto been called "resting." Actually it is apparent that this uniform low columnar epithelium is active in both secretion and absorption. When starch is fed, this appearance is most marked since the bulk of the diet ensures a low epithelium and there are no visible signs of absorption by the usual histological techniques. After feeding either water, gelatine (Plate 2, Fig. 10), or fat, conspicuous vacuoles appear in the distal cytoplasm and remain there for some hours. With the usual fixatives it is not possible to differentiate between the epithelia of *Blattella* fed any of these three substances.

The Golgi substance also reacts to the ingestion of foods. Excellent preparations of the Golgi substance in the *Blattella* midgut epithelium are obtained by the Mann-Kopsch technique. It is present as discrete granules in the characteristic pattern of the invertebrates, and shows well-marked changes correlated with the nutritional state and diet of the insects. The normal epithelial cell exhibits a large number of discrete granules mainly located towards the lumen but with a few granules proximal to the nucleus and some against the connective tissue (cf. Gresson 1934). In some cells there are conspicuous masses which presumably result from the clumping of a number of granules. When a cockroach is starved for 3 days the Golgi substance disperses and is found more or less evenly scattered throughout the cell. Aggregations as described in the normal insect are not found. If *Blattella* is starved for 3 days then fed starch, it is found that there is within half an hour a darkening of the whole cell, and conspicuous clumping of the Golgi substance occurs on the lumen side of the nucleus (Plate 5, Fig. 30). Feeding on gelatine also results in clumping of the Golgi substance, but the conspicuous dark staining cells are not present (Plate 5, Fig. 31).

We have presented reasons for the conclusion that the extrusion of cytoplasmic globules and fragments does not represent merocrine secretion. Shinoda (1927) has claimed, with little evidence, that secretion in *Blattella* is usually merocrine, but is holocrine following a period of starvation. This conclusion has been widely quoted (for example, Wigglesworth 1939, p. 264), but

has never been examined critically. Our evidence suggested that the hypothesis was questionable, and it was therefore studied further. If the excised midgut of *Blattella* is placed in acetic-orcein for a few minutes the epithelium can be removed from the connective tissue and muscularis. If the epithelium be then mounted and examined under an oil immersion lens the nuclei are found to be stained and the number of mitoses can be counted. The results, following a variety of treatments, are given in Table 6.

TABLE 6
NUMBER OF MITOSES PER 25 NIDI IN *BLATTELLA* MIDGUT

	Normal Controls	Starved 48 Hours	Starved 48 Hours, then Fed Starch			
			30 min.	60 min.	120 min.	300 min.
Mitoses per 25 nidi: means of 10 counts	4.3	2.6	1.6	3.0	4.6	4.3

There is rarely more than one mitosis per 5 nidi in the *Blattella* midgut, and the number may be as low as 1.6 mitoses per 25 nidi. Thus, taking the estimate of 40,000 nidi, there will be from 2500 to 8000 mitoses per midgut at any one time. If the mitotic process occupies about 60 minutes it would take from 120 hours at the minimum to 40 hours at the maximum replacement rate to regenerate all the epithelial cells in the adult *Blattella* midgut. While this indicates a fairly rapid rate of replacement, the increase is not sufficient to permit of a change to the holocrine mode of secretion. It is apparent that all that takes place is an accelerated rate of the normal sequence of cell division, growth, and regeneration.

(d) Summary of Cytology of Secretion

A synthesis of these results suggests a concept of the processes of secretion in the cockroach midgut which is probably also applicable to the caeca. The details are summarized in Figure 2. Each nidus is surrounded by a number of epithelial cells (about 10 or 12) which originate from it. The number of proepithelial cells in each nidus varies considerably. It is low in starvation, greater in a fed insect. Thus, epithelial replacement can occur either as a result of mitoses in the nidi (Fig. 2, A and B) or by maturation of nidus cells without simultaneous mitosis. The midgut epithelial cell is mature, that is, it functions in secretion and absorption, as soon as it reaches the midgut lumen. As it ages it is pushed farther away from the nidus — although this migration (A^1 to A^4 or B^1 to B^4) need never be greater than 2 or 3 cell widths. During the later stages of the development of the mature cell, granules form in the cytoplasm, and the cell becomes more sensitive to poisoning or to cytolysis caused by starvation or other factors. Next, globules of cytoplasm may be expressed through the striated border (as in cell B^3), a phenomenon hastened by many fixatives, and finally the nucleus and the remainder of the cytoplasm may be extruded into the gut lumen. During starvation, the mature cells continue to break down and are

replaced mainly from the cells already in the nidi. Some mitoses occur, but fewer than during feeding. Following the ingestion of foodstuffs (distilled water produces no effect) the number of mitoses is augmented, increasing the size of the nidi, but not necessarily the number of epithelial cells. Maximum enzyme output occurs from mature, but not from degenerating, cells.

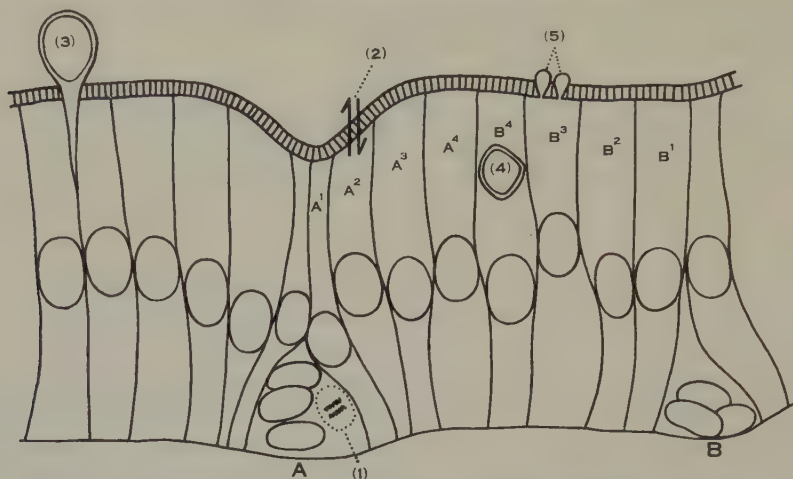


Fig. 2.—Diagram of epithelium of *Blattella* midgut showing sequence of changes occurring from the origin of the cells in the nidi, A and B, to degeneration (3). $A^1 - A^4$ and $B^1 - B^4$ represent cells of increasing age, which have originated from regenerative nidus A and regenerative nidus B, respectively. (1), a mitotic figure; (2) indicates that secretion of digestive enzymes and absorption occur in the same epithelial cell; (3), the final stage in cell degeneration showing extrusion of nucleus and adherent cytoplasm; (4), a cytoplasmic granule in an old cell; (5), cytoplasmic globules in process of being expelled through the striated border.

VI. DIGESTIVE ENZYME STUDIES

(a) Introduction

The data presented in the previous section indicated the cytological basis of enzyme secretion in *Blattella*, but a number of questions on enzyme secretion still require elucidation. We have attempted to obtain answers to the following questions:

- (i) Where in the gut are the digestive enzymes produced?
- (ii) Does the amount of enzyme change with different diets?
- (iii) Does the amount of enzyme change during starvation and subsequent feeding?
- (iv) Is enzyme production related to the intensity of epithelial regeneration in caeca and midgut?

The last question is considered in Section VII below.

To obtain data on the first three points, quantitative estimations of amylase, invertase, and proteinase have been made, using *Blattella* adults under a variety

of experimental conditions. A few similar experiments were performed on *Periplaneta*. The enzyme systems selected are all of fundamental importance in digestion. Purification of enzyme extracts was not attempted since it was not considered necessary for the purposes in view. The results are a measure of the capacity of the crude extract to break down starch, sucrose, and protein respectively.

(b) Methods

The tissues to be studied were removed from decapitated insects, freed from adhering tissues, and ground with a little sand. The suspension was diluted with glycerine-phosphate buffer mixture (Linderström-Lang and Duspiva 1936) to a volume depending on the method used, i.e. 1 midgut per ml. for colorimetric proteinase, 2 midguts per ml. for titrimetric proteinase, or 0.4 midgut per ml. for amylase and invertase. After standing with occasional stirring at room temperature for about an hour, the suspension was centrifuged to give a clear extract. When cells only were extracted, double this concentration was used, and in the studies on regional variation of the quantity of enzyme in the gut, the number of insects used was further increased by the number of regions into which they were divided, i.e. three for *Blattella* and four for *Periplaneta*. For the purpose of this experiment, *Periplaneta* was shown in preliminary experiments to have about six times the quantity of enzymes as *Blattella* and the extracts were diluted accordingly. For this reason no comparison of quantity of enzyme between the two species may be made.

Proteinase activity was estimated by two methods, titrimetric and colorimetric. The former was a modification of the Willstätter titration in alcohol, similar to Schlottke's (1937a) technique, with the exception that a gelatine substrate was substituted for casein as it was found to be more convenient. Two ml. of 6 per cent. neutral gelatine, 1 ml. of M/15 phosphate buffer pH 8.0, and 0.5 ml. of enzyme extract were mixed, then two 0.5 ml. samples were titrated immediately to a faint blue using thymolphthalein as the indicator with N/20 alcoholic (90 per cent.) potassium hydroxide; 4.5 ml. of absolute alcohol were then added and the mixture titrated to the final faint blue end-point, this titration representing the blank estimation. The enzyme-substrate mixture was held for 24 hours in a water-bath at 37°C., preserved with toluene, and the same titration procedure repeated. The difference between the titrations was transformed as follows. The relative concentration of serial dilutions from a strong enzyme extract gave a curve when plotted against the titration figures (Fig. 3). To permit comparison of results on different parts of the curve the relative enzyme concentration was arbitrarily divided into units and these were used to represent enzyme activity.

The colorimetric method employed was described by Charney and Tomarelli (1947) for the determination of proteolytic activity in duodenal juice. The substrate consisted of a solution of a chromophoric protein derivative (sulphanilamide azocasein), the digestion of which yields coloured components soluble in trichloroacetic acid. 0.5 ml. of substrate (pH 8.0) was mixed with 0.5 ml. of enzyme extract and held in a water-bath at 37°C. for 1 hr., after which 4 ml. of

5 per cent. trichloroacetic acid solution were added and thoroughly mixed. The unchanged substrate was filtered off and to 3 ml. of the filtrate were added 3 ml. of 0.5N sodium hydroxide to develop the colour. A blank estimation was made using the glycerine-phosphate solution instead of enzyme extract. The optical density (log absorption) was read on a Lange photoelectric colorimeter with a blue filter having a maximum transmission at about 480 m μ and a dilution curve for conversion to units of proteinase was constructed as described above (Fig. 3).

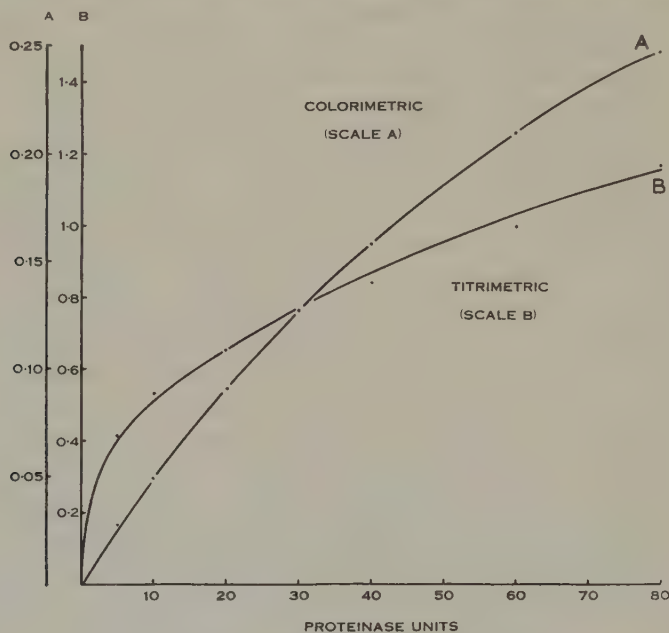


Fig. 3.—Dilution curves of *Blattella* proteinase.
Colorimetric method. Scale A. Optical density (log absorption).
For details of colorimeter, see text.
Titrimetric method. Scale B. Millilitres of N/20 alcoholic (90 per cent.) potassium hydroxide.

The Linderström-Lang and Holter (1933) technique was modified slightly for the estimation of amylase and invertase. A mixture of 5 ml. of 1.5 per cent. soluble starch, 2 ml. of M/15 phosphate buffer pH 6.5, 0.5 ml. of 0.2N sodium chloride, and 0.5 ml. of enzyme extract was used for amylase determination, and a similar mixture containing 5 ml. of 2 per cent. sucrose, 2 ml. of M/15 phosphate buffer pH 6.5, and 1 ml. enzyme solution for invertase. After 1 hr. in the water-bath at 37°C., 3 ml. aliquot samples were added to 2.5 ml. carbonate buffer at pH 10, and 1 ml. of N/10 iodine added. The tubes were stoppered for half an hour, the contents then acidified with 2.5 ml. of 2.4N sulphuric acid and the remaining iodine titrated with N/50 sodium thiosulphate. The differences between these titrations and those of boiled enzyme blanks were transformed by means of a dilution curve (Fig. 4) constructed as described for proteinase and these values were taken as a measure of amylase and invertase activity.

The results of all experiments (except those of Tables 7 and 10) are presented as activity per gut. When expressed as activity per unit weight the results are more variable owing to the large variable fraction of inactive gut contents. The statistical analyses were carried out on the individual titrimetric

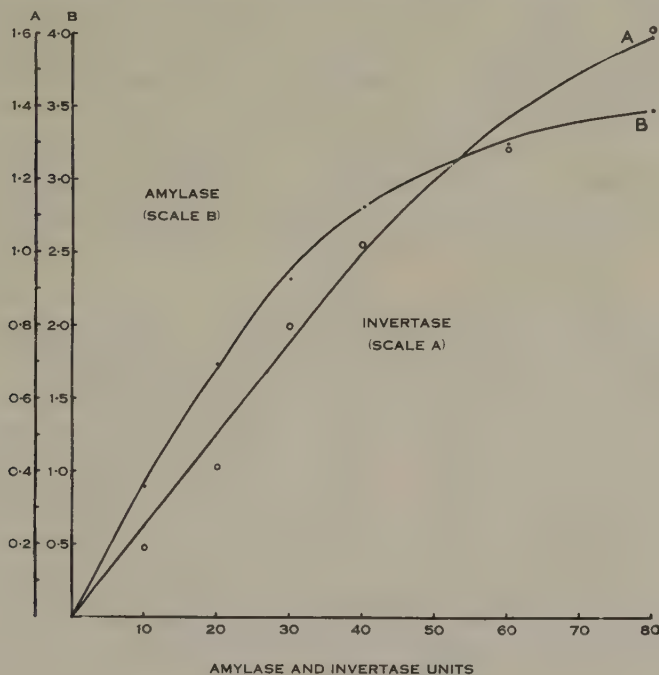


Fig. 4.—Dilution curves of *Blattella* invertase and amylase.
Invertase. Scale A. Millilitres of N/50 sodium thiosulphate.
Amylase. Scale B. Millilitres of N/50 sodium thiosulphate.

or colorimetric data, but the results are presented for the sake of clarity as average units (determined from the dilution curves).

Proteinase was determined by the colorimetric method in the midguts of ten male and ten female *Blattella* in order to detect any difference in amount of enzyme between the sexes (Table 7).

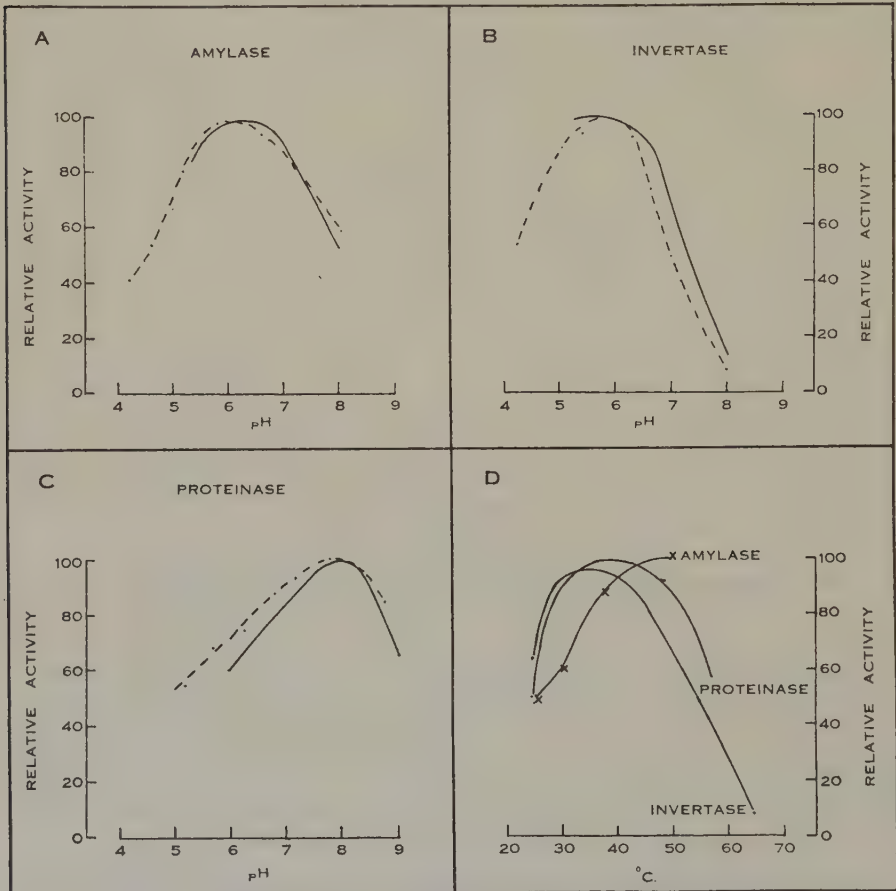
TABLE 7
PROTEINASE CONCENTRATION IN MALE AND FEMALE *BLATTELLA*

Male		Female	
Midgut Weight (mg.)	Proteinase (units)	Midgut Weight (mg.)	Proteinase (units)
2.09 ± 0.14	0.210 ± 0.022	3.32 ± 0.70	0.247 ± 0.044

The weight of the female midgut was more variable and on the average higher than for males, but the proteinase content was not greatly different. On the basis of this we made no distinction between the males and females in the subsequent experiments.

(c) *Enzyme Characteristics*

Of the characteristics of the enzymes, we studied the pH and temperature optima. Wigglesworth (1928) has shown that the pH optimum of *Blattella* amylase and invertase is about pH 6 (Figs. 5A and 5B) and that *Periplaneta* proteinase has an optimum about 7.8 (Fig. 5C) when acting on 5 per cent. gelatine. Two single experiments confirmed these results for amylase and invertase respectively. Another experiment (Fig. 5C) on *Blattella* proteinase showed that this had the same optimum as *Periplaneta* proteinase, i.e. pH 7.8-8.2.



----- DATA FROM WIGGLESWORTH (1927)

————— AUTHORS' DATA

Fig.5.—Characteristics of *Blattella* and *Periplaneta* digestive enzymes. See text for description.

The effect of temperature on the activity of the three enzymes is shown in Figure 5D. Since the temperature optimum is dependent on time of incubation it is not possible to compare proteinase with the other two enzymes, but it is

clear that in a period of 1 hour at 50°C. *Blattella* amylase is not inactivated as rapidly as invertase. When amylase extract in water was heated in a boiling water-bath for varying periods slight activity still remained after 2 minutes, but none after 5 minutes. Proteinase treated similarly had slight activity after 1 minute but none after 2 minutes.

Dialysis of the enzyme extract through a collodion membrane markedly reduced amylase activity, but had little or no effect on proteinase or invertase. Amylase activity was restored on addition of sodium chloride.

It will be observed that the data for both *Blattella* and *Periplaneta* are very similar, and no striking differences in the characteristics examined are apparent between the insect enzymes and those of vertebrates. These preliminary experiments were necessary in order to standardize conditions for the subsequent experiments.

TABLE 8
DISTRIBUTION OF DIGESTIVE ENZYMES IN REGIONS OF THE MIDGUT OF *BLATTELLA*

Gut Region	Amylase (units)	Invertase (units)	Proteinase (units)
Caeca	21.8	63.0	16.0
Anterior midgut	9.0	43.0	21.0
Posterior midgut	13.0	28.0	17.0

TABLE 9
DISTRIBUTION OF DIGESTIVE ENZYMES IN REGIONS OF THE MIDGUT OF *PERIPLANETA*

Gut Region	Amylase (units)	Invertase (units)	Proteinase (units)
Caeca	> 80	> 80	> 80
Anterior midgut	5	> 80	15
Mid midgut	11	67	6
Posterior midgut	5	40	3

(d) *Sites of Enzyme Production in Relation to Gut Morphology*

Preliminary experiments indicated that most of the amylase was produced by the salivary glands. Several experiments were designed to give information on the relative amounts of enzyme occurring in the cells in different regions of the midgut. In all experiments on *Blattella* the midgut was divided into caeca, and anterior and posterior parts. The cells alone, free of gut contents, were used for the midgut samples, but whole caeca were taken, since the contents of this part of the gut consist only of substances in solution. Owing to the much larger gut in *Periplaneta* it was possible to divide it into caeca, and anterior, mid, and posterior parts of the midgut.

In all instances the trends are fairly uniform within experiments. Thus in both species there was more amylase in the caeca than in the midgut. The concentration of invertase decreased from anterior to posterior in both species, but the drop was not marked. There is a difference between the two species with regard to the distribution of proteinase activity. Thus in three experiments

the concentration in all parts of the midgut of *Blattella* was similar, but in *Periplaneta* the caeca contained the bulk of the enzyme and the activity was considerably less in the posterior regions of the midgut.

These results cannot be correlated with differences in histological structure or with the absorptive propensities of the cells (see below). In Plate 1, Figures 1 and 4, it will be noted that the epithelial cells in the more posterior part of the *Blattella* midgut are more cuboidal than those in the anterior part, yet both produce equivalent amounts of proteinase. In *Periplaneta* the cytology is not greatly different in the fore and hind parts of the midgut, but the amount of proteinase produced is apparently greatly reduced in the latter region. Summarizing, it has been shown that amylase, invertase, and proteinase are present in extracts from all parts of the midgut, but the amount varies in different regions.

TABLE 10
PROTEINASE CONCENTRATION IN COCKROACH CROPS

	Weight of Sample (mg.)	Proteinase Activity (optical density)
7 full <i>Blattella</i> crops	23.6	0.010
10 full <i>Blattella</i> crops	40.4	0.007
1 full <i>Periplaneta</i> crop	98.6	0.530
1 empty <i>Periplaneta</i> crop	59.8	0.011
1 empty <i>Periplaneta</i> crop	25.3	0.057

In view of several reports in the literature of the presence of enzymes in the cockroach crop, we were surprised to find very little proteinase in the crop of *Blattella*. In an attempt to find whether there was a difference between *Blattella* and *Periplaneta* in this respect the proteinase activity in one full and two empty *Periplaneta* crops and in two groups of full *Blattella* crops was estimated by the colorimetric method. The results (Table 10) show clearly that proteinase is present in the full *Periplaneta* crop but not found in significant amounts in *Blattella*.

(e) Effect of Diet on Digestive Enzyme Production

We attempted to obtain data on this point by feeding *Blattella* pure starch, gelatine, or sucrose for varying periods.

(i) In the first series, *Blattella* were fed on pure gelatine, starch, or sucrose for 3 days. These were compared with roaches starved for 3 days and with others fed the normal food which consisted of cut potato and bran mash. After feeding, 4 midguts from each group were extracted, made up to 10 ml., and the invertase and amylase activity determined. A similar series of tests, except that sucrose was omitted, was carried out for proteinase using the titrimetric technique. For these latter, midgut extracts of each group of 4 *Blattella* were made up to 2 ml. The results given in Table 11 demonstrate that amylase activity is high in *Blattella* fed a normal diet, but is considerably reduced by starvation for 3 days. Feeding gelatine or sucrose also reduces it, but to a slightly less degree than starvation. A starch diet for 3 days *decreases* the amylase activity

to less than one-tenth the normal value. The activity of invertase and proteinase is not greatly changed following any of the above treatments.

TABLE 11

CHANGES IN ACTIVITY OF AMYLASE, INVERTASE, AND PROTEINASE IN THE MIDGUT OF *BLATTELLA* ON VARIOUS DIETS

	Amylase (units)	Invertase (units)	Proteinase (units)
Fed normal diet	25.0*	32.4	12
Starved 3 days	4.6	25.4*	14
Fed gelatine 3 days	7.4	49.5	21
Fed starch 3 days	1.7*	43.8	22
Fed sucrose 3 days	6.6	32.3	—

* Indicates the differences are significant.

(ii) In the second series, 3 groups of 4 insects were used for each treatment, which consisted of feeding, after 3 days' starvation, the same types of foodstuff as before for periods ranging from a few minutes up to one hour. Each gut was separated into 3 parts, (a) crop, (b) cells of midgut, and (c) contents of midgut, and amylase and invertase activity determined (Tables 12 and 12A).

TABLE 12

CHANGES IN ACTIVITY OF AMYLASE AND INVERTASE IN DIFFERENT REGIONS OF *BLATTELLA* GUT AFTER FEEDING FOR SHORT PERIODS. ACTIVITY OF AMYLASE AND OF INVERTASE EXPRESSED AS UNITS

Amylase Activity

	Starved 3 Days	Starved then Fed Starch		Starved then Fed Gelatine		Starved then Fed Sucrose	
		10 min.	1 hr.	10 min.	½ hr.	10 min.	1 hr.
Crop	3.3	44.0	21.0	10.0	9.0	4.0	11.0
Midgut cells	1.9	3.0	2.5	3.0	2.0	3.0	1.5
Midgut contents	3.8	3.0	2.5	4.0	3.5	1.5	2.0

Activity in the crop after feeding is significantly higher than that of the starved insects. None of the other differences are significant.

TABLE 12A

Invertase Activity

	Starved 3 Days	Starved then Fed Starch		Starved then Fed Gelatine		Starved then Fed Sucrose	
		10 min.	1 hr.	10 min.	½ hr.	10 min.	1 hr.
Crop	1.2	1.5	1.0	2.3	3.0	1.0	0
Midgut cells	11.0	10.0	7.0	8.5	9.0	12.0	8.0
Midgut contents	19.0	7.0*	15.0	13.0	17.0	5.0*	12.0

* Indicates differences are significant.

Changes in proteinase activity were studied by the colorimetric technique in a separate experiment, using the same food materials except for sucrose.

Proteinase in the entire midgut of five individual insects was estimated for each treatment (Table 13).

Considerable variation can occur between individuals even within a treatment. Thus with the small numbers used only very marked differences between treatments are significant. Amylase increases only in the crop, and this increase is greater after feeding with starch than with either sucrose or gelatine (Table 12). This may be due to the quantity of food ingested (which was greater, as determined by a comparison of weights) or to the consistency of the food, since the greater part of the enzyme is secreted by the salivary glands. There is some amylase in the midgut cells, but whether this is an endoenzyme or due to contamination has not been determined.

Practically no invertase is present in the crop. The treatments cause no change in invertase activity in the midgut cells, but the activity in the contents falls after feeding with starch or sucrose. Both of these diets cause a rise in invertase activity during one hour. From Table 13 it is evident that there is a

TABLE 13
CHANGES IN PROTEINASE ACTIVITY PER WHOLE MIDGUT OF *BLATTELLA* AFTER
FEEDING. ENZYME ACTIVITY EXPRESSED AS UNITS

Fed normal diet	36	
Starved 3 days	23	
	Fed Starch	Fed Gelatine
Starved, then fed $\frac{1}{2}$ hour	17	18
Starved, then fed $1\frac{1}{2}$ hours	21	15*
Starved, then fed 3 hours	20	22
Starved, then fed 6 hours	24	26
Starved, then fed 24 hours	27	28

* Indicates the difference is significant.

drop in proteinase activity following starvation and that this is accentuated after feeding gelatine. However, the concentration returns almost to normal within some hours after feeding.

These results suggested that three days' starvation may have an adverse effect on the insects. An experiment was therefore designed to show any reduction in the ability of *Blattella* starved for 3 days to regain their normal enzyme activity. Two groups of 6 control *Blattella* were fed normally for 6 days. Two other groups were starved for 3 days and then fed the normal diet for 3 days. Proteinase activity in midgut extracts was estimated by the colorimetric method. The results (Table 14) indicate that the weights of the midguts from the insects which had undergone 3 days' starvation are lower than the controls, and that, even allowing for the considerable decrease in weight, the amount of proteinase is still lower. This must be kept in mind when drawing conclusions from Tables 12 and 13.

The following general conclusions can be drawn from the data presented in this section.

- (1) In general, a decrease in the production of digestive enzymes results from starvation.
- (2) All enzymes are secreted irrespective of the nature of the food.
- (3) The enzyme activity is reduced following the feeding of its substrate and recovery is comparatively slow. Schlottke (1937*b*) arrived at the same conclusion as a result of his work on *Periplaneta*. The continuous feeding of *Blattella* on starch markedly depletes the salivary amylase.
- (4) There are some quite marked differences between *Blattella* and *Periplaneta* as reported by Schlottke (1937*b*), and in part confirmed by us.

TABLE 14
EFFECT OF 3 DAYS' STARVATION ON WEIGHTS AND PROTEINASE ACTIVITY OF
BLATTELLA MIDGUT

	Weight of Gut Tissue (mg.)	Proteinase Activity (units)
Starved 3 days,	6.7	36
then fed 3 days	6.3	29
	8.6	46
Normal controls	7.8	46

VII. STIMULATION TO SECRETION

It was shown in the previous section that some digestive enzymes increase in concentration in insects which are fed following a period of starvation. There are several possible mechanisms whereby this increased secretion might be stimulated: (*a*) the foodstuff itself or its products may stimulate secretion; (*b*) the stimulus may be nervous, or (*c*) the stimulus may be hormonal. All or any of these factors may be involved as, in fact, they are in vertebrates.

(*a*) It is difficult to prove the action of a secretagogue in insects, but there are several pertinent lines of evidence. For example:

- (1) Distilled water alone has no detectable effect on secretion in *Periplaneta* (Table 15).
- (2) The caeca of this species may reach considerable lengths (about 14 mm.), and are ill-fitted for the rapid diffusion of materials from the gut lumen to their closed ends.
- (3) Schlottke (1937*b*) claimed that the caeca start to secrete before the midgut. This suggests that the caeca receive the stimulus to secrete before the midgut.
- (4) However, they are not well innervated (see below) and, if they respond to a stimulus it could only be carried in the caecal contents, by the haemolymph, or through the tracheae. The latter route seems very unlikely, not only because no such mechanism seems to have been employed by insects, but also because of the anatomical relations of the tracheal trunks.

The above evidence is insufficient to decide whether stimulation of the caeca and midgut is mediated by secretagogues.

(b) The vital methylene blue technique, modified from Kuwana (1935), was found to demonstrate the innervation of the *Periplaneta* and *Blattella* alimentary canal. The stock rongalit solution, made up in distilled water and used concentrated, was injected into both species until they were distended. Optimum staining was obtained after 3 hours at room temperature (about 18°C.). The tissues were dissected, immersed in a saturated aqueous solution of ammonium molybdate, and the spreads studied under high dry and oil immersion objectives. The same insects which showed excellent staining of the nerves, nerve endings, and nerve cell bodies in the crop showed no trace of nerves in the caeca. The epithelial cells of both caeca and midgut were, however, outlined in surface view by fine, blue granules which, under high magnification, could be seen to be confined to the region of the striated border. No trace of methylene blue stained bodies could be seen towards the proximal end of the cells. If the caeca are innervated, their nerves must be refractory to methylene blue. An occasional cell body and a few extremely fine nerves were found in the best preparations of the *Periplaneta* midgut. These nerves were entirely superficial and almost certainly innervated only the muscularis. The salivary glands, on the other hand, were extremely well innervated, being completely enveloped by a fine mesh of nerve fibres. While nerve cell bodies could be seen on the adjacent pharynx in this region none was found on the salivary glands. Only an occasional very fine nerve could be found innervating the salivary reservoirs.

In *Blattella* the caeca were devoid of visible nerves, but the midgut was quite conspicuously innervated over its entire length. In contrast, the salivary glands were comparatively poorly supplied with nerves. Thus *Blattella* and *Periplaneta* are quite different in regard to the innervation of their respective midguts and salivary glands. In both cases, however, it was concluded that the nerves to the midgut innervated the muscularis, and could not be concerned with the stimulation of the epithelium. This conclusion was strengthened by the results of the use of the classical silver staining techniques for nerve endings. We obtained successful impregnation of nerves in the crop by the Hortega method, but better results were obtained by Boeke's technique (see Lee, p. 578). Plate 3, Figures 14 and 15, shows sections from the same slide of caecum and crop. Nerves are visible running between and into the epithelial cells of the latter, but there is no trace of them in the caeca. Bodian's protargol method did not demonstrate nerves in *Blattella* or *Periplaneta* although it gave excellent preparations of some other insects.

The above data indicate that the stimulus to secretion of the caecal and probably of the midgut epithelia is not mediated by nervous impulses, in spite of the importance of this type of stimulus in vertebrates. The time relations of the responses support this conclusion.

(c) There remains the possibility that a factor, liberated into the haemocoel and carried by the haemolymph, influences the secretion of the midgut epithelium.

We have found in the literature no previous attempt to examine this hypothesis, and we were not able to test it for *Blattella* because of the small size of this species. The following experiments were, therefore, performed on *Periplaneta*.

We considered that the rate of regeneration of midgut cells, as indicated by the mitoses in the nidi, would provide data on the physiological state of the midgut. If the whole midgut of *Periplaneta* is dropped into acetic orcein for ten minutes, and the caeca cut off, their contents and the epithelium can be squeezed on to a slide. The epithelium from each caecum is then covered with a 1 in. by 1 in. coverslip which is pressed down firmly with blotting paper. The number of mitoses in the regenerative nidi can then be counted under a high dry objective and gives a measure of the regenerative activity of the caeca. Plate 5, Figure 26, shows a nidus and mitotic figure. The midgut itself is more difficult to study in

TABLE 15
NUMBER OF MITOSES IN 25 NIDI IN CAECA OF *P. AMERICANA*. EACH FIGURE IS
THE MEAN OF 10 COUNTS

Starved 7 days	1 Hour	2 Hours	4 Hours
<i>Period after feeding artificial food</i>			
2.5	3.7	7.0	4.6
<i>Period after feeding distilled water</i>			
	5.6	2.9	3.9
<i>Period after feeding cut potato</i>			
6.5	5.4	9.5	6.3

this way since the regenerative cells are arranged in long anastomosing rows, but the number of mitoses per oil immersion field can be estimated. In each caecum 10 counts are made of the number of mitoses per 25 nidi. In the normal feeding cockroach the number is approximately 8. In an experiment to determine the relation between mitotic index and enzyme production in a series of insects three caeca were used for the estimation of proteinase and three from the same insects for mitotic counts. The results suggested that there is probably a weak inverse correlation between cell replacement by mitotic activity in the nidi and enzyme concentration, although the relationship is not precise. This is rather to be expected, in view of the fact that the enzymes are probably reduced from cells some time after they have undergone mitosis (see previous section).

A lag between stimulus and response was demonstrated in Schlottke's (1937*b*) data on the effects of feeding on enzyme production. Such a lag could not be expected on the basis of a neural mechanism, so an attempt was made to determine whether there was any mitotic response within a few hours to ingested material. The results (Table 15) were variable and showed no significant difference between treatments, indicating that there is probably no detectable stimulation in *Periplaneta* within 4 hours.

These results were, however, suggestive, so experiments were designed to provide evidence for the possibility of the existence of a factor in the blood of a feeding *Periplaneta* which would affect the mitoses in the caeca. Blood from

feeding *Periplaneta* was removed from a neck puncture, sucked into a glass needle, and injected into the coxo-femoral joint of a starved individual. The technique employed by Yeager and Munson (1945) was found satisfactory, but unless the operation was performed rapidly the blood coagulated and clogged the needles before the injection could be completed. Clean needles were employed for each injection. Insufficient blood could be obtained from starved *Periplaneta* for a control to be employed to demonstrate that the effect was not due to a factor in the blood itself unconnected with feeding. The results (Table 16) gave some indication that there may be an effect of the injected blood. But the data are variable and the technique is not easy.

TABLE 16
NUMBER OF MITOSES IN 25 NIDI IN THE CAECA OF *P. AMERICANA*. EACH FIGURE IS THE MEAN OF 10 COUNTS

Normal Fed (A) Donors of Blood	Starved 7 Days (B)	One Hour after Blood from (A) Injected into (B)	Two Hours after Blood from (A) Injected into (B)
7.1	3.7	4.8	4.3

Difference significant at 5 per cent. level, 1.6.

Difference significant at 1 per cent. level, 2.9.

The difficulty of obtaining statistically significant results with *Periplaneta* led to the search for more satisfactory material, and attention was directed to the Coleoptera, in some groups of which the regenerative cells are contained in crypts which extend into the haemocoel. (For a description of the crypts of *Dytiscus*, see Duspiva 1939.) It was found that mitoses could be readily counted in the crypts of *Tenebrio molitor* if small segments of the orcein stained midgut were examined under the oil immersion objective. In the adult *Tenebrio* there are about 3000 crypts. Those of the anterior and mid parts of the midgut are larger than those of the posterior region. A section of crypt from the *Tenebrio* midgut is illustrated in Plate 5, Figure 27.

Tenebrio molitor has the additional advantage over *Periplaneta* that the food passes almost immediately into the midgut, instead of being retained for considerable periods in a storage crop, as it is in the Blattidae.

In all experiments with *Tenebrio*, counts of the number of mitoses in 10 crypts from sections of the gut at the anterior end, the mid part, and the posterior region of the midgut were made. The number of individuals per treatment varied from 2 to 6 in different experiments, but was usually 4.

Firstly, to determine whether the results might be affected by different periods of the day at which counts would be made, *Tenebrio* were fixed every 2 hours from 8 a.m. to 4 p.m. The results (Table 17) indicated the absence of a detectable diurnal rhythm.

Some idea of the frequency of mitoses and the time required for the mitotic cycle was obtained from the study of colchicine injected *Tenebrio*. Normal insects were injected with about 0.1 ml. of 0.5 per cent. colchicine in 0.7 per cent. saline solution. This produced toxic symptoms within one hour and the mitoses in

many of the caeca were difficult to count. After 2 hours many of the crypts were very reduced in size. The injection of saline alone produced no discernible effects.

TABLE 17

MEAN NUMBER OF MITOSES IN EACH OF 30 CRYPTS FROM THE MIDGUT OF *TENEBRIO MOLITOR*. ALL INSECTS FED IN SEPARATE CONTAINERS

8 a.m.	10 a.m.	Noon	2 p.m.	4 p.m.
3.9	4.2	4.0	4.5	4.0

TABLE 18

MEAN NUMBER OF MITOSES IN EACH OF 30 CRYPTS FROM THE MIDGUT OF *TENEBRIO*

Normal Fed Controls	Time after Injection of Colchicine				
	15 min.	30 min.	60 min.	120 min.	240 min.
4.5	3.3	5.6	4.2	7.2	8.6

From these data it will be seen that, under the conditions of the experiment, mitoses occurred at the rate of about 1 per hour. Further, it can be calculated by the method employed by Leblond and Stevens (1948) that the duration of mitosis is about 2 hours. This is slow compared with the mitotic rate in *Drosophila*, for example (cf. Sonneblick 1948), but is of the correct order of magnitude. (It should be mentioned that attempts to use the colchicine method on *Blattella* or *Periplaneta* were not successful. In these insects colchicine decreases rather than increases the number of mitoses, indicating that it does not inhibit the functioning of the spindle, but merely appears to poison the mitotic process. This is unexpected in view of the success of a number of authors to inhibit spindle formation in orthopteran gametogenesis.)

The results of various periods of starving and subsequent feeding on *Tenebrio* are given in Table 19.

TABLE 19

MEAN NUMBER OF MITOSES IN EACH OF 30 CRYPTS FROM THE MIDGUT OF *TENEBRIO*

Normal Fed	Period of Starvation			
	24 hr.	48 hr.	72 hr.	96 hr.
3.8	3.5	3.3	2.6	2.5

Starved 96 Hours, then Fed Potato						
½ hr.	1 hr.	1½ hr.	2 hr.	2½ hr.	3 hr.	4 hr.
2.7	2.6	2.9	3.1	3.5	3.4	4.1

Difference significant at 5 per cent. level, 0.71.

It is evident that the effects of starvation are marked after 3 days, and that the effect of feeding increases up to 4 hours. If the beetles are allowed to feed for 4 hours only and are then examined daily, it is found that the effect of feeding persists for 24 hours but is not present after 48 hours (Table 20).

TABLE 20
MEAN NUMBER OF MITOSES IN EACH OF 30 CRYPTS FROM THE MIDGUT OF
TENEBRIO MOLITOR

Normal Fed	Starved 3 Days	Starved, then Fed Potato			
		4 hr.	24 hr.	48 hr.	72 hr.
3.3	2.4	4.1	4.4	3.3	3.3

Difference significant at 5 per cent. level, 0.81.

The effects of feeding gelatine or starch, rather than potato, showed that both diets had a similar effect on the number of mitoses (Table 21). While stimulation is induced by either diet the histological effects of these two diets are dissimilar. The intercryptal epithelium is normal following starch, but greatly reduced following gelatine feeding.

TABLE 21
MEAN NUMBER OF MITOSES IN EACH OF 30 CRYPTS FROM THE MIDGUT OF
TENEBRIO MOLITOR

Controls Normal Fed	Starved 48 Hours	Starved 48 Hours, then Fed Gelatine			Starved 48 Hours then Fed Starch		
		½ hr.	2 hr.	6 hr.	½ hr.	2 hr.	6 hr.
4.6	2.9	3.5	2.1	3.7	3.4	2.1	3.5

Difference significant at 5 per cent. level, 0.84.

It will be observed that the period of maximum mitotic activity follows some hours after feeding. However, this is the normal *response* which is a slow one, and the stimulus may be expected to have exerted its effect some time previously. The critical experiment to determine the presence in the blood of a factor affecting midgut mitoses, was made by comparing the effect of blood from starved or from fed *Tenebrio* when injected into starved individuals.

After trying several methods the injections were most satisfactorily performed by allowing blood, obtained from an adult *Tenebrio* by puncture of the intersegmental membrane in the cervical region, to form a drop on a clean microscope slide. This drop, of approximately 50 λ , was immediately drawn into a fine glass needle into which the shortened shank of a No. 20 hypodermic needle was fixed by shellac. The advantages of a glass needle are: (1) that the amount of blood can be regulated visually, and (2) that there is no question of the completeness of the injection. Injection was made into the haemocoel through the soft abdominal tergum normally concealed beneath the elytra.

Blood from a fed insect causes a slight rise in the number of mitoses in the midgut crypts after 30 minutes. Blood from a starved insect causes no such rise (Table 22). This experiment was repeated with the same results, and together they strongly suggest the presence of a factor in the blood of fed, but not of starved, *Tenebrio*, which affects the number of mitoses in the midgut crypts.

After normal feeding, the response does not become evident for about 1½ hours and increases up to 2½ hours (see Table 19 above). After the injection

of blood from a fed individual, however, the response is more rapid, and, in addition, is transient as is shown in two separate experiments (Table 23).

TABLE 22
MEAN NUMBER OF MITOSES IN EACH OF 30 CRYPTS FROM THE MIDGUT OF
TENEbrio MOLITOR

Normal Fed Controls (A)	Starved 3 Days (B)	Blood from (B) Injected into (B) after ½ hour	Blood from (A) Injected into (B) after ½ hour
3.7	2.3	2.2	2.9

Difference significant at 1 per cent. level, 0.5.
Difference significant at 0.1 per cent. level, 0.7.

TABLE 23
MEAN NUMBER OF MITOSES IN EACH OF 30 CRYPTS FROM THE MIDGUT OF
TENEbrio MOLITOR

Fed Donors	Starved 2 Days	Time after Injection of Blood from Fed Donors into Starved Individuals			
		½ hr.	1 hr.	2 hr.	4 hr.
3.3	2.3	3.6	2.6	1.6	2.8

Difference significant at 5 per cent. level, 1.0.

Controls Starved 4 Days (A)	Half an hour after Injection of Blood from Fed Insects into (A)	One hour after Injection of Blood from Fed Insects into (A)
2.9	3.9	2.3

Difference significant at 5 per cent. level, 0.4.

There is no question, therefore, that the response following injection differs from that of the normal insect fed after a period of starvation. In spite of this there is strong evidence that a factor in the blood of a normal feeding *Tenebrio* is able to affect the rate of mitosis in the midgut crypts. We shall refer to the factor as the "midgut regeneration stimulating factor" (M.R.S.F.).

It is clear that the injection type of experiment is not as well suited to an investigation of this kind as parabiotic experiments, but neither *Periplaneta* nor *Tenebrio* is amenable to such operative techniques.

(d) *Source of the M.R.S.F.*—A number of sources for the production of the M.R.S.F. may be considered: (i) the corpora allata and cardiaca, (ii) the salivary glands, and (iii) the epithelial cells of the midgut.

(i) The innervation of the corpora allata and cardiaca differs from that of most organs in that nerves are received from the supraesophageal ganglion and also from the hypocerebral ganglion which is part of the stomodeal nervous system, and is in neural connection with the frontal ganglion. This latter ganglion innervates the pharynx and buccal cavity, and is in a position to receive sensory

impulses arising as a result of feeding. It was thought, therefore, that the corpora allata or cardiaca, which are obviously endocrine organs, may be involved in the production of a M.R.S.F. This hypothesis gains credence from the suggestion that the corpora allata are involved in moulting and metamorphosis (Wigglesworth 1939), and both these processes are usually accompanied by great increases in mitotic activity in the midgut (Henson 1946). Plate 3, Figure 18, illustrates the results of this mitotic activity in a moulting nymph of *Blattella*.

It is known that fuchsinophilic secretory granules occur at times in the corpora cardiaca. But no correlation could be found between their presence in *Periplaneta* and the nutritional state of the insect. It would appear that the corpora cardiaca have other functions, but no evidence has been brought forward that they are active in the production of the M.R.S.F. Wigglesworth's (1948) indication that food is digested more rapidly in *Rhodnius* females under the influence of the hormone affecting egg maturation may be significant in this connection.

(ii) The salivary glands are, like the corpora allata and cardiaca, innervated from the stomatogastric nervous system. The glands contain two cell types, but it is not known whether they are both secretory and whether they both produce constituents of the saliva.

(iii) The cells of the alimentary tract itself may produce the M.R.S.F., since in vertebrates they produce gastrin, etc., but data on the origin of the M.R.S.F. in insects await further research.

Summary.—It will be clear from the above experiments that secretion in the insect midgut may be initiated either by the action of secretagogues or by a hormone. Evidence for the former is not yet available, but suggestive evidence is presented in favour of the latter hypothesis. A neural stimulus does not seem to be involved.

VIII. THE ROLE OF THE ALIMENTARY CANAL IN ABSORPTION

Ingested food may be either in a condition for direct absorption through the gut wall, or may be digested to such a state by the enzymatic processes discussed in the previous sections. The permeability of various parts of the alimentary tract of any insect has never been fully investigated and it is not known, for example, whether proteins may be absorbed directly, or whether they must be completely broken down to amino acids before absorption.

Many authors have claimed that fat is absorbed directly through the cockroach crop (Abbott 1926; Petrunkewitch 1900; Scharrer 1947), though how this occurs is still obscure. The tracheae were at one time considered to play an active part but this theory has been discounted (Schluter 1912). Absorption of fat also occurs in the midgut and caeca (Scharrer 1947).

The absorption of inorganic ions, for example, those of iron, has likewise been widely studied. In fact, a number of conclusions regarding the absorptive cells in insects have relied on the localization of iron absorption as their sole evidence.

Our studies have indicated, not only that different substances may be absorbed in different regions, but also that considerable differences may be found even between insects belonging to a single family. Data will be presented on absorption of the following substances:

- (a) Cations, Fe^{++} , Fe^{+++} , Ba^{++} , Sr^{++} , Cu^{++} .
- (b) Anions, H_2PO_4^+ .
- (c) Ascorbic acid.

(a) *Cations*.—The distribution of a number of cations in insect tissues has been studied in this laboratory by D. F. Waterhouse, who has kindly permitted the quotation of his unpublished results (for methods, see Waterhouse 1940*b*). The distribution of these ions in the alimentary tracts of *Blattella* and *Periplaneta* is given in Table 24.

TABLE 24
DISTRIBUTION OF ANIONS IN ALIMENTARY CANAL OF *PERIPLANETA* AND *BLATTELLA*

	Crop	Caeca	Midgut	Anterior Hindgut	Mid Hindgut	Rectum
<i>Periplaneta</i>						
Normal food	—	Fe^{+++}	—	—	Fe^{+++}	—
Metal enriched food	—	$\text{Fe}^{+++}\text{Fe}^{++}$	Fe^{+++}	—	$\text{Fe}^{+++}\text{Fe}^{++}$ Ba^{++}	—
<i>Blattella</i>						
Normal food	—	—	—	—	$\text{Fe}^{+++}\text{Fe}^{++}$	—
Metal enriched food	—	Fe^{+++}	Fe^{+++}	—	$\text{Fe}^{+++}\text{Fe}^{++}$ Ba^{++}	—

It is to be especially noted that absorption in the hindgut is confined to a band of cells, not as clearly defined as the iron cells of *Lucilia* (Waterhouse 1940*b*), but nevertheless to a quite well-defined zone. Ferric iron is invariably found in high concentration in this region of both *Periplaneta* and *Blattella*. This ion is usually found also in the caeca in *Periplaneta*, but not in *Blattella*.

Absorption of copper could not be demonstrated either in *Periplaneta* or *Blattella*. When very large amounts are ingested from enriched foods barium may be detected in the caeca of the midgut of *Periplaneta* and of *Blattella* and strontium in the caeca of *Periplaneta* and in the hindgut of *Blattella*.

These data are more complete than those of previous authors who have studied the absorption of cations in the insect gut. It is clear that there are marked differences even between insects of a single family, but the reasons for these differences are still quite unknown.

(b) *Anions*.—We attempted to study the absorption of phosphate by feeding the dibasic sodium salt. When the monobasic salt was added to the diet the roaches died, although Lindsay and Craig (1942) used it without these effects in their studies of the absorption of radiophosphorus. Equal quantities of the sodium dihydrogen phosphate and the artificial diet were mixed thoroughly

and moistened with a small amount of water. Sections were cut and treated in the usual manner for controls for the Gomori alkaline phosphatase technique. In normal *Blattella* no phosphate is demonstrated in the midgut by this method and only a small amount in the hindgut. After feeding on the above diet for four days there are small accumulations in the distal regions in the caecal epithelium, a smaller, but still detectable amount in the same regions of some midgut epithelial cells (mainly in the anterior half of the midgut), and very considerable quantities in the epithelium of some regions of the hindgut. Since, however, there is frequently phosphate in these hindgut cells it is difficult to determine whether it is absorbed in this region. Lindsay and Craig's (1942) data suggest that it is not.

If *Blattella* is fed *d*-glucoascorbic acid, the amount of phosphate in the hindgut is greater than in normal insects (cf. Plate 3, Fig. 19, and Day 1949a).

(c) *Ascorbic Acid*.—Prior to the recent investigation by Day (1949b) no studies had been reported on the histological localization of ascorbic acid in insect tissues. It is now known to occur in a wide variety of tissues and in some tissues of practically all insects studied. In the *Blattella* gut ascorbic acid occurs normally only when the insects are fed on a diet containing it. If the culture is maintained on an ascorbic acid free diet and then transferred to a diet rich in it, differences in its absorption in different regions of the midgut become apparent. Since details have been published recently (Day 1949b) it is sufficient to mention that both midgut and caeca absorb ascorbic acid in *Periplaneta* and *Blattella*. The hindgut does not appear to be involved in the absorption of this material.

It has been indicated that the caeca of *Blattella* would seem to be better suited to the function of secretion than to that of absorption. However, they absorb ascorbic acid, and there is other evidence that they may be more efficient in absorption than might appear: (i) small quantities of phosphate and iron may also be absorbed by the caecal epithelium, (ii) when dyes are introduced into the alimentary canal, as in the hydrogen ion experiments previously considered, the dyes always fill the caecum lumen, and (iii) the absorption of the products of starch digestion as evidenced by the dark cells following the Mann-Kopsch Golgi technique is as conspicuous in the caeca as in the midgut (Plate 5, Fig. 30).

The two general conclusions from these studies on absorption are: (1) that different substances are absorbed in different regions of the gut of a single species of insect, and (2) that the same substance may be absorbed in two different regions of the gut of different species even though they be closely related. Gresson's (1934) conclusion (based on the use of iron saccharate) that the forepart of the midgut of *Periplaneta orientalis* is mainly secretory while the posterior part is mainly absorptive, is not substantiated with *P. americana* or *B. germanica*.

IX. THE FUNCTION OF THE ALIMENTARY CANAL IN INTERMEDIARY METABOLISM

The examination of some of the substances concerned in intermediary metabolism cannot provide an indication of the multitudinous processes occurring following the absorption of foodstuffs. We have studied the distribution of glycogen, alkaline phosphatase, and lipase in the *Blattella* gut, as a preliminary essay in this field.

(a) *Glycogen*.—No study of the distribution of glycogen in the cockroach gut is known to us, and there are in fact a number of disagreements in the literature regarding its presence in the insect alimentary canal (see Yeager and Munson 1941; Babers 1941, for reviews of the literature). Difficulties with methods are undoubtedly the cause of these differences of earlier workers, and indeed the Bauer, Best's carmine, and iodine methods do give capricious results even in the hands of experienced investigators. A recent technique (Gomori 1946) in which tissues are incubated in silver methenamine solution, has given uniformly good results with *Blattella* tissues, and may be considered specific if studied in conjunction with controls treated with salivary ptyalin. (The only structure in the insects we have studied which still gives a positive reaction in saliva treated controls is the peritrophic membrane of *Lucilia cuprina* larvae.)

An unexpected distribution of glycogen in the *Blattella* midgut has been found. While a small amount is present throughout the midgut (Plate 2, Fig. 12), a ring of cells near the anterior end in the region of the proventriculus is particularly rich in glycogen (Plate 2, Fig. 11). The distribution in the cells is characteristic. The greater part of the glycogen is just above the nuclei. There is an area of lower concentration below the striated border and the latter gives an intensely positive reaction. Some of the cell inclusions apparently contain some glycogen. It is particularly interesting that the cells rich in glycogen are in the same region as those showing the argentophil granules described above. However, the distribution in the cells is different and the two substances are obviously not the same. Wigglesworth (1942) has observed glycogen (iodine method) in some clearly defined regions of the *Aedes* midgut, but most of it was in the posterior half.

From a study of the midguts of *Blattella* which have been either fed a normal diet, starved for 3 days, fed starch 3 days, or fed gelatine 3 days, it is clear that the glycogen in the midgut is fairly constant in quantity. Yeager and Munson (1941) conclude that "the gut-cell glycogen varies with the intake of carbohydrate," but our data do not substantiate this hypothesis. In fact the starch fed insects had conspicuously less glycogen in the midgut than the other treatments (Plate 2, Fig. 13). Starving for 3 days does not appear to deplete the stainable glycogen of the midgut to any observable extent.

(b) *Alkaline Phosphatase*.—The occurrence in the gut of phosphatase with an optimum at pH 5.0 has been mentioned on p. 181. Drillhon and Busnel (1945) have reported that phosphatase active at pH 9.5 is present in the alimentary tract of certain insects, and Bradfield (1946) has located this enzyme histochemically in the midgut of *Cossus*, using the technique of Gomori (1939). The results of

the application of this method to the tissues of a number of insects have recently been reported (Day 1949a). In *Blattella* the enzyme is present in the foregut, hindgut, and rectum, but it is particularly concentrated in the circular muscles at the anterior end of the midgut. The functional significance of these results is by no means clear. A variety of diets and experimental treatments failed to influence the distribution and this fact validates the study of the sites of absorption of phosphate as described in the previous section.

(c) *Lipase*.—Of the digestive enzymes, a method has been described only for the detection of lipase (Gomori 1946). This method has been used on *Blattella* with success. The enzyme appears to be labile, although satisfactory preparations have been obtained when all operations were performed with a minimum of delay.

In normal *Blattella*, lipase is found to be fairly uniformly distributed through all midgut epithelial cells, but it is perhaps slightly more abundant in those of the regenerative nidi than in the mature cells. It was not found in the epithelium of the crop, which is perhaps surprising in view of the evidence that the crop can absorb fat (see Section VIII). It is more abundant in the hindgut. In this location, but not in the midgut, the intensity of the reaction appears to be somewhat lower following a prolonged fat diet.

X. HISTOPATHOLOGY OF INSECTICIDES

It may be expected that the study of the effect of appropriate substances on the alimentary canal will elucidate certain details of the physiology of this organ. We have attempted to investigate the histopathology of insecticides with particular attention to the incipient changes in the midgut epithelium. First it was necessary to differentiate between necrosis and effects which are attributable to the specific action of the insecticides. We therefore studied a series of *Blattella* killed by (a) decapitation, (b) heat, (c) carbon dioxide, and (d) cyanide.

(a) Twenty-four hours after decapitation the insects may still move their appendages violently when stimulated, but conspicuous changes are found in the midgut. These probably represent incipient necrotic changes and are characterized by increasing numbers of cytoplasmic inclusions, increasing nuclear acidophily, and finally destruction of the striated border with cytolysis (Plate 4, Fig. 20).

(b) After killing by heat (56°C. for 15 min.) the cells of the midgut epithelium become separated and present a characteristic form of granular disintegration of the cytoplasm with clumping of the chromatin leading to karyorrhexis (Plate 4, Fig. 21). After 60 minutes at the same temperature the striated border is still conspicuous, but the nuclei have lost their ability to stain differentially.

(c) Carbon dioxide acts very rapidly as an anaesthetic to *Blattella*, but they recover quickly even after a 3 hour exposure to it. After a 6 hour exposure only a small percentage recovers. Insects fixed after this treatment show only slight cytoplasmic vacuolization in the anterior part of the midgut. However, in

the posterior part of the midgut cytoplasmic globules, expressed through the striated border, increase in number and may become very numerous (Plate 4, Fig. 23). The globules frequently are vacuolated. Cell breakdown may become so extensive as to produce a crenulated appearance in the epithelium like that of *Periplaneta*.

(d) *Blattella* is rapidly killed by exposure to potassium cyanide. After a 3 hour exposure recovery never occurs. The midgut of such an insect is considerably damaged. Cytoplasmic globule formation is frequent in the anterior and posterior parts of the epithelium and whole cells may be discharged into the lumen (Plate 4, Figs 24 and 25). The globules are more deeply staining than those produced by the effects of carbon dioxide.

With these preliminary data the histology of the epithelium of *Blattella* treated with several insecticides was examined. The insecticides were incorporated in the artificial diet, and although some food was ingested they could have been absorbed through the cuticle. Three types of effects were observed: (i) marked epithelial breakdown by the arsenic compounds, (ii) slight epithelial changes by chlordane, BHC, and "Ryanex,"* and (iii) almost no effect by DDT.

(i) Sodium arsenite was studied in *Periplaneta* in relation to so-called "hypersecretion" (see Section V). The effects in *Blattella* are essentially the same. One hour after injection a number of the oldest epithelial cells show the early signs of cytoplasmic globule formation (Plate 3, Fig. 17), while after 2 hours many cells have discharged their cytoplasm, resulting in some places in the complete disorganization of the epithelium. It should be noted especially that it is the oldest cells farthest from the nidi which degenerate first.

(ii) Poisoning by chlordane results in the loss of the striated border, separation of some epithelial cells, and incipient cytolysis. Conspicuous dark brownish deposits are found in the distal region of the epithelial cells and acidophilic inclusions are frequent in the cytoplasm proximal to the nucleus. BHC and "Ryanex" produce vacuoles in the cytoplasm, the circular muscles contract and become conspicuous but otherwise the midgut is unaffected (Plate 4, Fig. 22).

(iii) DDT produces a tendency towards cytoplasmic globule formation but has remarkably little effect on the midgut.

All insecticides examined, other than sodium arsenite, appear to exert their lethal action with little visible change in the midgut, even though this organ of *Blattella* undergoes striking histological changes. This fact gives a special interest to those substances which do produce distinctive changes in the midgut. Sodium arsenite, for example, certainly seems to hasten cell breakdown in *Blattella* as it does in *Vanessa* and *Locusta* (Pilat 1935) and *Prodenia* (Woke 1940). Further work in this field of insect pathology is most desirable.

* "Ryanex" described as "100% production grind for dusts" kindly supplied by Merck & Co. Inc.

XI. DISCUSSION

Some of the data presented in the previous sections are at variance with current concepts and others represent new aspects of the processes of digestion in insects. These will be discussed in the following paragraphs.

(a) It is sometimes considered that *Blattella* and similar insects are discontinuous feeders, in contrast to many other insects, particularly larvae, which are able to ingest almost continuously. Crowell (1943) has shown that even in the larva of *Prodenia* there are several periods of non-feeding per hour which almost equal the actual feeding time. But the alimentary tract is certainly always kept full of food. Our observations on the rate of passage of foodstuffs through the gut of *Blattella* indicate that in this species also the midgut is normally kept full of food and a mechanism for the stimulation of secretion at the time of feeding may therefore not be required. The continued production of digestive enzymes during starvation and the damaging effect of 3 days' starvation on *Blattella* provide further evidence for the fact that this species, while it may ingest food in a discontinuous manner, is not a discontinuous feeder in the usual sense. Food passes through the alimentary tract of *Blattella* at a considerably greater rate than it does through that of *Periplaneta* (Snipes 1938).

(b) The reducing conditions demonstrated in the hindgut of *Blattella* would be presumably more difficult to maintain in the presence of an abundant supply of oxygen. It is, therefore, interesting to note the poorer tracheation of the large intestine in comparison with that of the midgut (Plate 5, Fig. 29) and of the rectum, in the contents of which the reducing conditions are not maintained. The information is not yet available for a more detailed comparison between redox conditions and tracheation in the insect gut, but the problem would be worthy of future study.

(c) The correlation of histological structure with secretory activity is not the simple situation which has frequently been described in the insect midgut. The precise nature of many cell inclusions is still unknown, but there is no justification for describing "secretion" or "absorptive" inclusions without more information than can be obtained by histological or histochemical techniques. The hypothesis of Shinoda (1927) that the midgut epithelium of *Blattella* produces enzymes normally by merocrine secretion, but by holocrine secretion after starvation, has been shown to be untenable. In its place we have a concept of the replacement of worn out cells by regeneration from the nidi, while the main burden of both secretion and absorption is carried on by the epithelial cells previously referred to as "resting." This hypothesis is strengthened by the critical study of the cytology of the midgut epithelium and the observation that the cells which degenerate are always the old cells far from the regenerative nidi. Henson (1929) and certain earlier authors, on morphological evidence, arrived at similar conclusions.

Steudel (1912) concluded from his studies that the absorption of iron and fat occurred in the epithelial cells previously described as resting. We concur with this opinion and from our enzyme studies we are able to go one step further,

for we have proved that cells in this same condition are also active in secretion. The conclusion follows that in *Blattella*, secretion and absorption go on simultaneously from and into the same cell. This is in distinction to a widely accepted hypothesis that epithelial cells go through a cycle, being first absorptive and later secretory in function. Gresson (1934) concludes that both secretory and absorptive cells occur in the caeca and in the anterior part of the midgut, but maintains that secretion and absorption never take place in the same cell. Steudel (1912), in common with the majority of authors, described secretory stages which are probably degenerating cells.

The rapidity of the renewal of the insect midgut epithelium indicates the intense activity characteristic of this tissue. Few other animal tissues are provided with such well-defined nidi of embryonic cells. But this short life of the midgut epithelium is not confined to insects, for Leblond and Stevens (1948) have calculated that the duodenal epithelial cell of the albino rat lives only about 36 hours.

The general conclusions from these data are that both secretion and absorption can go on without visible cytological changes, and that both processes go on in epithelial cells as soon as they reach the lumen of the gut. We have demonstrated an approximate inverse relationship between the mitoses in the regenerative nidi and proteinase activity. We would not expect this relationship to be close since we have produced strong evidence that the most actively secreting cells are considerably older than those which undergo mitosis. There are very few data in the literature on the rate of regeneration of the midgut. Hodge (1937) reported that the number of mitoses per section almost doubled in *Melanoplus* (6.4 to 11.6) when the insects were fed on an incomplete diet as compared with a satisfactory one. These figures are difficult to evaluate since Hodge reported that the number of nidi increased on the unsatisfactory diet. However, they suggest compensatory hypertrophy of the epithelium attempting to cope with the unsatisfactory diet.

(d) The demonstration of digestive enzymes in the crop by both Abbott (1926) and Schlottke (1937), and confirmed in our experiments, raises the question of their origin. Our study of the time relationship between the appearance of proteinase in the crop in relation to its secretion into the caeca and midgut confirms the theory that the enzymes are regurgitated. A mechanism for this is obvious in the striking movements of the oesophageal invagination which appear to result in a sucking action in the isolated alimentary canal in saline. Wigglesworth (1930) has stressed the hypothesis that the function of this invagination is to permit the peritrophic membrane to arise anteriorly to the point of entry of the food into the gut. Moreover, he maintained that the peritrophic membrane is "pressed" between the invagination and the midgut wall. This may well be true in, for example, Diptera and in Dermaptera where there is a conspicuous annular ring on the posterior edge of the invagination which might well act as a press. But it certainly does not appear to be the case in *Blattella* or in *Periplaneta*. In these Orthoptera the distance between the

oesophageal invagination and the midgut epithelium is very many times the thickness of the peritrophic membrane. Moreover, the invaginated organ undergoes normal writhing movements which would render it unsuitable as a press. Consideration of these two points, together with the laminated structure of the peritrophic membrane, suggests that the complex structure of the invagination serves other purposes, and one of these is undoubtedly to regurgitate digestive enzymes from the midgut into the crop. However, the morphology of the organ is not dissimilar in *Periplaneta* and in *Blattella*. Yet we have shown that the *Periplaneta* crop may contain proteinase while it does not appear to be regurgitated into the *Blattella* crop. Certainly some additional function must be ascribed to the oesophageal invagination. A function, and an important one, of the crop is to become dilated with air during moulting to enlarge the cuticle, before hardening occurs. A study of the crop in moulting insects reveals that it is the main organ distended with air and that there must be an effective mechanism preventing the air passing into the midgut. The oesophageal invagination must also serve this purpose.

(e) While we have presented evidence for the presence in the blood of a fed *Tenebrio* of a factor resulting in increased mitoses in the midgut crypts, we have no data on the relationship between mitoses and enzyme formation in this species.

The evidence for the lack of a neural mechanism for the stimulation of secretion is anatomical. But it should be stressed that the innervation of the gut varies considerably, even within the Orthoptera. Nesbit (1941) has shown that the stomodeal nervous system may innervate the caeca in some species and even the midgut in, for example, *Rhomalea*. The study of species sufficiently large to permit artificial stimulation of these nerves will be necessary to decide whether they are simply motor in function.

It is not expected that all insects (continuous feeders, for example), would have a mechanism for stimulating midgut activity. A discontinuous feeder amenable to parabolic experiments should provide suitable material for the continuation of the study of the problems associated with the initiation of secretion.

(f) During the course of these investigations it has become apparent that there are marked differences between the details of the digestive physiology of the two cockroaches, *Blattella* and *Periplaneta*. These differences are as follows: (i) the innervation of the midgut and salivary glands; (ii) the arrangement of the regenerative nidi in the midgut; (iii) the distribution of glycogen; (iv) the distribution of invertase and proteinase production in the midgut; (v) the presence of proteinase in the crop; (vi) the period during which food remains in the crop (compare our results with those of Snipes 1938); (vii) the extent of enzyme concentration changes during starvation (compare our results with those of Schlottke 1937b); and (viii) the presence of invertase in the *Blattella* salivary glands and its absence in those of *Periplaneta* (Wigglesworth 1927).

The few data on each of the aspects of the physiology of digestion which we have presented above, when integrated with previous work, do not yet outline a comprehensive theory of the digestive processes of *Blattella*. They serve rather to indicate the lacunae in our knowledge, but advances are difficult and can only be expected as specialized microtechniques become available.

XII. ACKNOWLEDGMENTS

The work described in this paper was carried out as part of the research programme of the Division of Economic Entomology, C.S.I.R. The authors are greatly indebted to many of their colleagues for assistance in the preparation of this paper, and especially to A. T. James, Section of Mathematical Statistics, C.S.I.R., for performing the statistical analyses.

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EXPLANATION OF PLATES 1-5

PLATE 1

Midgut of *Blattella germanica*.

All photomicrographs were taken with Zeiss Ibo attachment under oil immersion x 96 objective, x 8 ocular, magnification x 580. Sections 10 microns. Alcoholic Bouin fixation and Mallory's Triple stain unless otherwise specified.

Figs. 1-4.—Longitudinal sections of one midgut, showing changes in cell size and shape in different regions of the gut. Figure 1 is in the region of the stomodaeal invagination. Note cytoplasmic granules. Figure 2 slightly posterior to the stomodaeal invagination. Note the characteristic arrangement of the cells in the regenerative nidus. Figure 3 is about two-thirds the length of the midgut from the oral end; and Figure 4 is just anterior to the point of entry of the malpighian tubules. Note shorter striated border and laminated peritrophic membrane in Figures 3 and 4.

Figs. 5, 6.—L.S. midgut; Bodian technique. Figure 6 shows conspicuous argentophil inclusions, mainly distal to the nucleus, but some proximal to it. Figure 7 is slightly caudad to Figure 6 and shows the complete absence of argentophil inclusions, and of the dense band just beneath the striated border. The indentations at the proximal side of the nuclei, characteristic of alcoholic Bouin fixation, are conspicuous.

PLATE 2

Details as for Plate 1.

Fig. 7.—Midgut of individual starved 3 days; showing cytoplasmic globules expressed through striated border. The high columnar epithelium is characteristic of individuals lacking gut contents.

Fig. 8.—Midgut of individual starved 2 days. A nucleus and surrounding cytoplasm are extruded into the midgut lumen. This is rarely found in fed insects.

Figs. 9, 10.—Effects of feeding. Fig. 9.—The active epithelium associated with absorption and high enzyme output, following starch ingestion for 3 hours. Note the relatively large number of cells in the regenerative nidi. The epithelium is stretched owing to the bulk of the diet. Fig. 10.—After feeding gelatine for 3 hours. The vacuoles distal to the nucleus are similar to those following ingestion of distilled water. Since gelatine is less bulky than starch the gut is less distended and the epithelial cells are more columnar.

Figs. 11-13.—Glycogen (Gomori technique) in *Blattella* midgut. Striated border is intensely stained. Fig. 11.—Normal individual — glycogen rich region. Fig. 12.—Normal individual — glycogen poor region. Note positive cytoplasmic granule. Fig. 13.—Starch fed individual — glycogen rich regions. Note less glycogen than in Figure 16.

PLATE 3

Alimentary canal of *Blattella* and *Periplaneta* — various techniques.

Fig. 14.—T.S. *Blattella* caeca, Boeke technique. No trace of nerves innervating the epithelial cells. x 580.

Fig. 15.—L.S. crop on the same slide, showing nerves (at A) running between epithelial cells. x 580.

Fig. 16.—T.S. *Periplaneta* caecum, one hour after injection of sodium arsenite. Incipient cell breakdown visible at (A). x 355.

Fig. 17.—The same after 2 hours — “hypersecretion” evident at the apices of most groups of cells. x 205.

Fig. 18.—L.S. *Blattella* midgut. At time of last ecdysis, but before there are externally visible signs of moulting. The nymphal epithelium is greatly vacuolated. Beneath it there is a layer of horizontally disposed cells, and beneath that is the adult midgut epithelium of which the cells are not yet mature, and many of them are in mitosis. x 355.

Fig. 19.—L.S. small intestine of adult *Blattella* fed *d*-glucoascorbic acid. Gomori phosphate stain, showing considerable deposit of inorganic phosphate in these cells. x 580.

PLATE 4

Histopathology of *Blattella* midgut. Alcoholic Bouin fixation and Mallory's Triple stain. All x 580.

Fig. 20.—Twenty hours after decapitation. Note incipient necrosis increased cytoplasmic inclusions. Also apparent in the original is nuclear acidophily and incipient destruction of the striated border.

Fig. 21.—The effect of heat (15 minutes at 57°C.). Note separation of the epithelial cells, marked basophilia, and granular cytoplasm.

Fig. 22.—The effect of the insecticide, “Ryanex”—the production of conspicuous vacuoles in the cytoplasm.

Fig. 23.—The effect of 6 hours exposure to carbon dioxide. Posterior part of the midgut showing incipient cytolysis and many cytoplasmic granules expressed through the striated border. Note crenulation of epithelium.

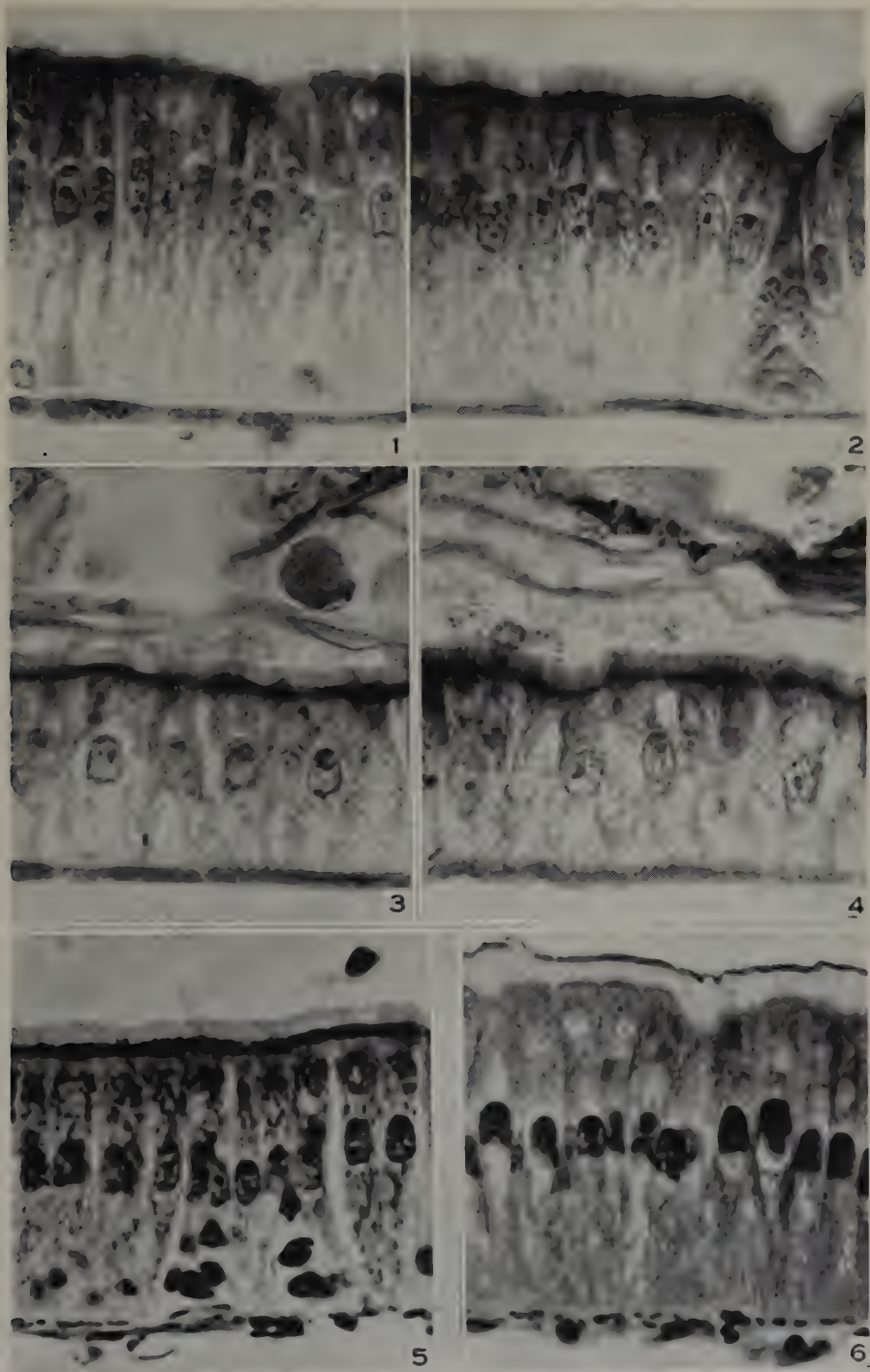
Fig. 24.—The effect of 3 hours exposure to potassium cyanide fumes. Conspicuous cytoplasmic globule formation and chromatic granules in the cytoplasm.

Fig. 25.—L.S. of a caecum of the same insect. Epithelial cells distorted towards midgut attachment of caecum. Cytoplasmic fragments, some containing nuclei, abundant in lumen.

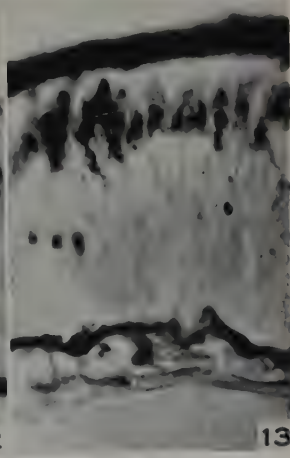
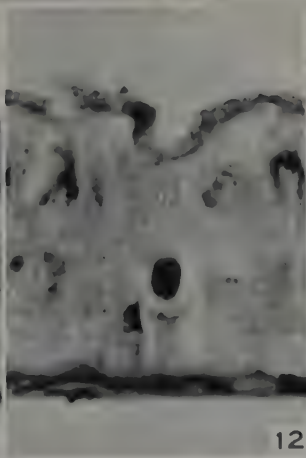
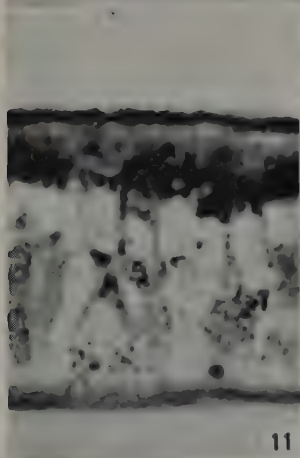
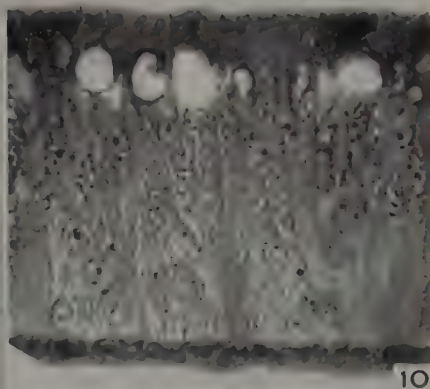
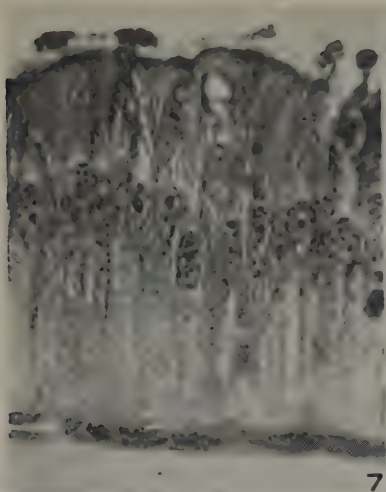
PLATE 5

Alimentary tracts of *Periplaneta*, *Blattella*, and *Tenebrio* — various techniques.

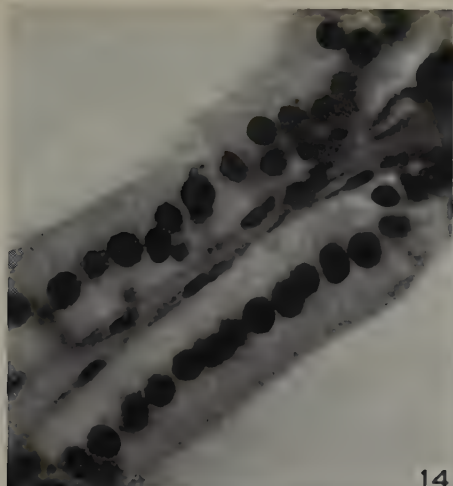
Fig. 26.—Regenerative nidus from spread of *Periplaneta* caecum, stained in acetic orcein, and showing mitotic figure.



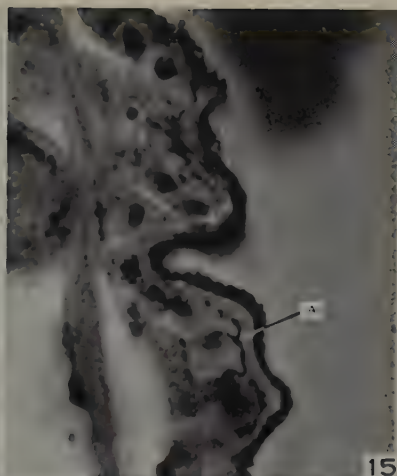
DAY AND POWNING.— A STUDY OF THE PROCESSES OF DIGESTION IN CERTAIN INSECTS



DAY AND POWNING.— A STUDY OF THE PROCESSES OF DIGESTION IN CERTAIN INSECTS



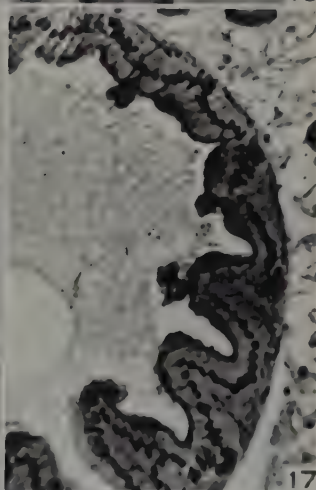
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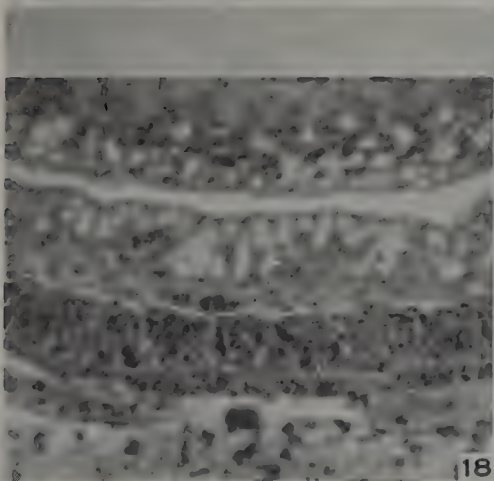
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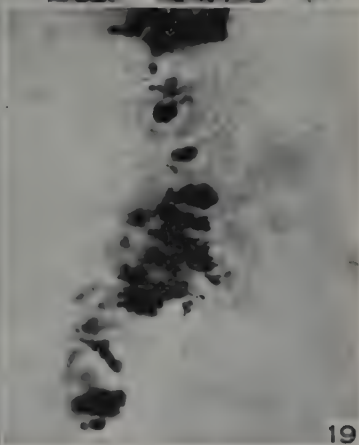
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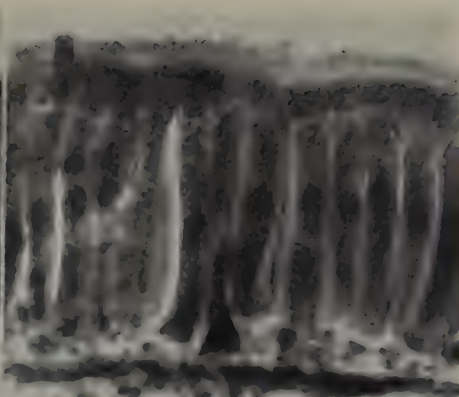
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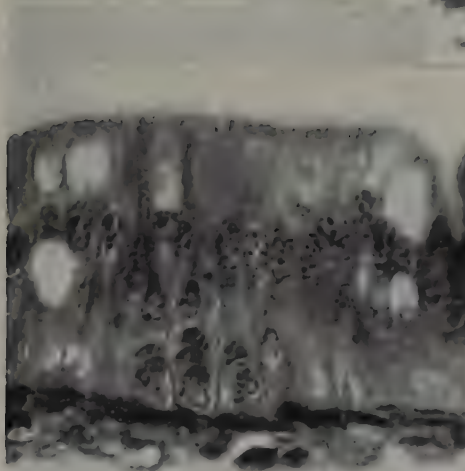
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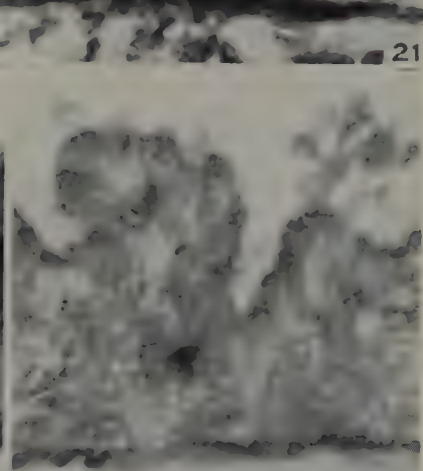
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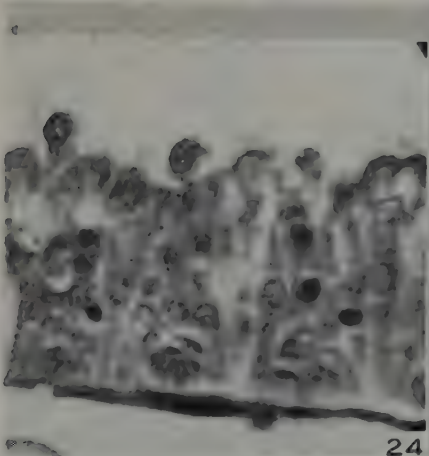
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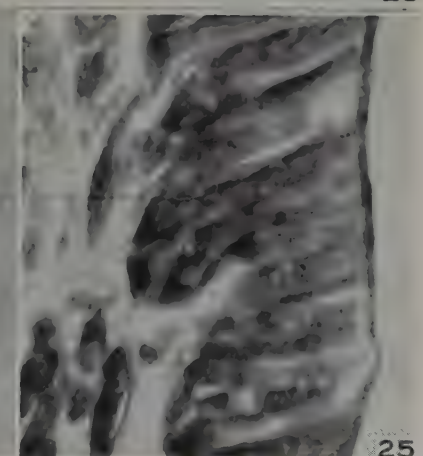
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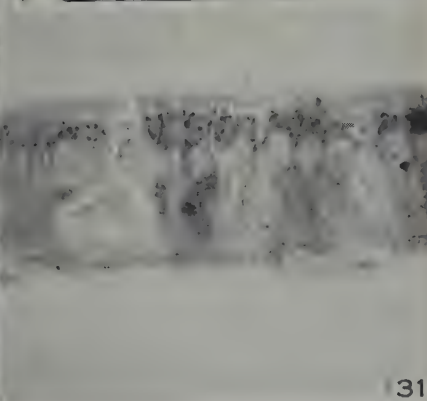
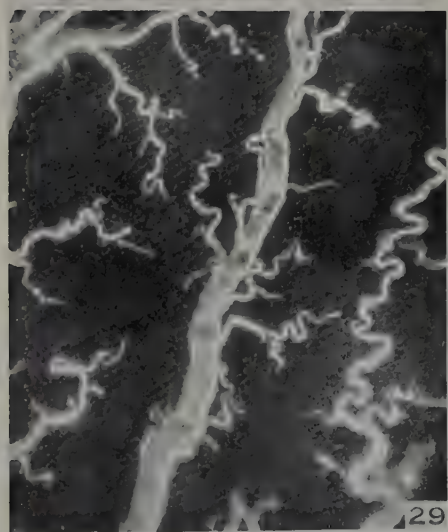
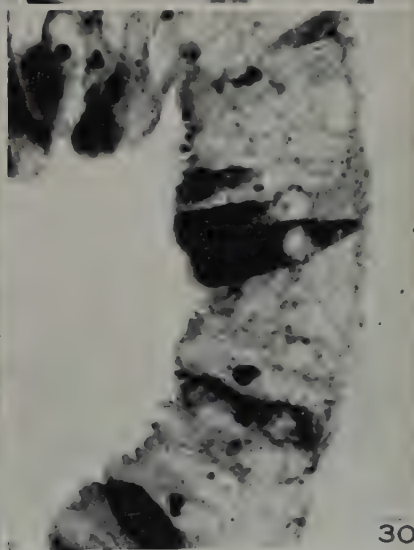
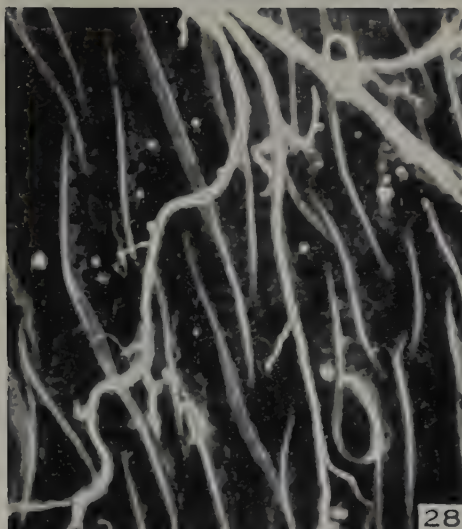
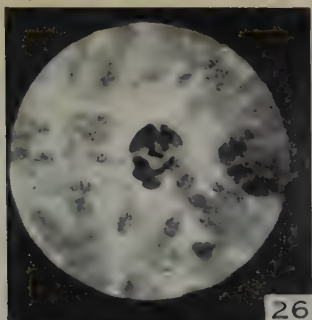
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DAY AND POWNING.— A STUDY OF THE PROCESSES OF DIGESTION IN CERTAIN INSECTS

- Fig. 27.—L.S. *Tenebrio* midgut showing midgut contents on the right, cuboidal intercryptal epithelium and section of regenerative crypt. The regenerative cells are at the blind end of the crypt. Fat body at extreme left. Bodian technique. x 130.
- Fig. 28.—Spread of *Blattella* crop under dark field showing distribution of tracheae and some tracheoles. x 355.
- Fig. 29.—The same of midgut showing large tracheal trunks and characteristic end twigging. Every epithelial cell is tracheolated. x 355.
- Fig. 30.—T.S. Caecum of *Blattella* fed starch 3 days, Mann-Kopsch technique. Golgi substance clumped towards lumen side of nucleus. Note dark-staining cells found after feeding on starch. x 355.
- Fig. 31.—L.S. Midgut *Blattella* fed gelatine 3 days, Mann-Kopsch technique, showing characteristic position of Golgi substance in feeding individuals. x 355.

THE ACTION OF NINHYDRIN ON THE ENZYMATIC AND ACTIN-COMBINING PROPERTIES OF MYOSIN

By D. GILMOUR* and J. H. CALABY*

[Manuscript received April 4, 1949]

Summary

Ninhydrin at 5 μ M per mg. myosin produces 90 per cent. inhibition of myosin-ATP-ase at pH 9.0 in 3 hours at 5°C. At pH 7.0 the same inhibition is produced by 35 μ M ninhydrin per mg. myosin.

This inhibition is reversed by cysteine, the release being complete after one hour's treatment of the myosin by ninhydrin, but incomplete, except with very high cysteine concentrations, after three hours' treatment of the myosin. Other amino acids did not release the inhibition produced by ninhydrin.

The addition of actin to myosin treated with ninhydrin at concentrations which produced over 90 per cent. inhibition of the ATP-ase resulted in a rise in viscosity similar to that encountered with untreated myosin. The addition of ATP to ninhydrin-treated actomyosin, however, usually caused a precipitation of the actomyosin rather than a dissociation into myosin and actin.

The removal of -SH groups of the myosin by ninhydrin was demonstrated by the disappearance of the nitroprusside reaction, but attempts to titrate -SH quantitatively in the presence of ninhydrin were unsuccessful.

I. INTRODUCTION

The association of the muscle proteins myosin and actin to form a complex characterized by high and anomalous viscosity has been studied by Szent-Györgyi and his colleagues (for reviews see Szent-Györgyi 1945, 1947). The addition of small amounts of adenosine triphosphate (ATP) to the actomyosin complex dissolved in 0.5M KCl has been shown to result in a drop in viscosity due to dissociation into the myosin and actin components. Recently Bailey and Perry (1947) have demonstrated that the presence of -SH groups on the myosin is necessary for the combination of myosin with actin, and that the same -SH groups are connected with the adenosine triphosphatase activity. Using ninhydrin as an -SH reagent, we have been able to demonstrate an inhibition of enzyme activity at concentrations which do not prevent the interaction of myosin and actin.

There is some evidence in the literature that ninhydrin has a strong affinity for -SH groups. Brückmann and Wertheimer (1947) have shown that both ninhydrin and the structurally related alloxan cause the disappearance of reduced glutathione from the blood of rodents. Alloxan is a recognized -SH reagent, and has been used to inhibit a number of enzymes (Lehmann 1939; Hopkins, Morgan, and Lutwak-Mann 1938; Naganna and Narayana Menon 1948; Walsh

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and Walsh 1948). Lieben and Edel (1932) suggested an interaction between alloxan and thiol groups, and Lazarow, Patterson, and Levey (1948), and Patterson, Lazarow, and Levey (1949) have shown that alloxan oxidizes cysteine and apparently forms an addition compound with the -SH of glutathione and protein. Recently Griffiths (1949) has shown that both alloxan and ninhydrin inhibit muscle hexokinase, and that this inhibition is released completely in the case of alloxan, partially in the case of ninhydrin, by cysteine. Zittle (1948) has demonstrated inhibition of ribonucleinase by ninhydrin (90 per cent. at 0.01M), but ascribes the effect to a reaction between ninhydrin and the amino groups of the enzyme.

II. METHODS

(a) Preparation of Myosin, Actin, and ATP

Crystalline myosin, prepared from rabbit muscle by the method of Szent-Györgyi (1945), was used in most of the enzyme tests and all the viscosity measurements. For some enzyme tests actomyosin, thrice precipitated by the method of Bailey (1942), was used. Glass-distilled water only was used in all enzyme preparations. Actin was prepared by the method of Straub (1943). ATP was isolated as the dibarium salt by Kerr's (1941) method, either from fresh rabbit muscle or from a dehydrated powder prepared from sheep muscle by the method of Szent-Györgyi (1947). Before use it was purified and converted to the sodium salt by being passed through a column of Amberlite IR-100, by the method recommended by Polis and Meyerhof (1947).

(b) Determination of Phosphorus and Nitrogen

Inorganic phosphorus was measured by the method of Fiske and Subbarow (1925). Nitrogen was determined by nesslerization after sulphuric acid digestion, using the reagent of Vanselow (1940). Weight of protein was calculated from the nitrogen estimations by using Bailey's (1942) figure for the nitrogen content of myosin, and Straub's (1943) figure for the nitrogen content of actin.

(c) Enzyme Tests

For tests of ATP-ase activity myosin was dissolved in 0.02M veronal-HCl buffer containing 0.5M KCl, of pH either 9.0 or 7.0. Neither glycine nor borate buffers could be used in inhibition studies with ninhydrin, as they reacted with the inhibitor. In one series of tests in which $\text{Na}_2\text{CO}_3\text{-NaHCO}_3$ buffer was used, results obtained were similar to those with veronal-HCl buffer, but the ATP-ase activity was lower. Enzyme activity was measured at 37°C. The test mixture usually contained 3 ml. of a myosin solution containing 0.12-0.43 mg. protein per ml., 1.5 ml. of an ATP solution containing about 0.28 mg. 7 min. P per ml., and 0.15 ml. of 0.1M CaCl_2 . Samples of 1 ml. volume for the estimation of inorganic P were withdrawn at intervals, usually at 5, 10, and 30 minutes after the commencement of incubation. At least three phosphate determinations were made for every enzyme test, although comparisons of activity were all made on the basis of the inorganic P produced in five minutes. In all tests the concentration of myosin was the rate-determining factor, and was such that usually about half

the ATP available was decomposed in five minutes. Myosin was fairly unstable at 37°C. in veronal buffer, particularly at the higher pH. In our experiments all the available ATP was never broken down; the curve of inorganic P production flattened out after 80-90 per cent. of the ATP had been used up, and at this time it was usually seen that the myosin had precipitated out. Unlike Mommaerts and Seraidarian (1947), however, we obtained higher activities at pH 9.0 than at pH 7.0, although the difference was not great.

The Q_p (cf. Bailey 1942) of crystalline myosin in our preparations varied from 800 to 2400, and that of actomyosin, prepared according to Bailey, varied from 400 to 1000. We confirmed the finding of Polis and Meyerhof (1947) that fresh myosin preparations tested against purified ATP showed no enhanced activity in glycine buffer, and no activation by cysteine. A stock solution of myosin (about 10 mg. protein per ml.) kept a few days at 5°C., however, lost some activity, which could be restored by cysteine. This loss of activity was hastened in dilute solutions. Myosin at 0.25 mg. per ml. stored at 5°C. lost up to 30 per cent. of its activity in 24 hours.

Ninhydrin, cysteine, and other agents to be added to the enzyme were dissolved immediately before use in 0.02M veronal buffer containing 0.5M KCl, the solutions being adjusted to the appropriate pH, 7.0 or 9.0, before being mixed with the enzyme solution. In testing the release of inhibition, ninhydrin was first allowed to act on the enzyme for varying lengths of time, after which the agent to be tested for release was added to the enzyme-ninhydrin mixture and allowed to act for approximately as long as the ninhydrin had acted alone.

(d) *Viscosity Determinations*

Viscosities were measured in Ostwald viscometers equilibrated in a water-bath at 7°C. Since the thixotropic property of myosin causes a gradual decrease in the time of outflow with successive readings, the first reading was used in the calculation of viscosities. The figures for specific viscosity quoted must thus be regarded as approximations only. However, since the changes we were concerned with were of considerable magnitude, any uncertainties due to the limitations of the Ostwald technique did not affect our conclusions.

The viscosities of a myosin solution and of the same myosin solution treated with actin were measured simultaneously in two viscometers. The actomyosin solutions were prepared by mixing 0.5-1.1 ml. of a solution containing 2.7 mg. of actin per ml. with 10 ml. of various myosin solutions containing 0.35-0.70 mg. per ml., the final ratio of myosin to actin being about 5:2 by weight in each instance. The effect of ATP on the viscosity of actomyosin was then measured after the addition, by means of a capillary pipette, of a small amount of ATP solution (0.3 ml., 0.2 mg. 7 min. P per 10 ml. actomyosin solution) to the viscometer containing actomyosin.

III. INHIBITION OF THE ATP-ASE

Inhibition of ninhydrin was studied first at pH 9.0. It was found that the inhibition was established rather slowly at 5°C. (Fig. 1). After this time relation had been established, inhibition studies were made after pretreatment with

ninhydrin for three hours at 5°C. The relation between concentration of inhibitor

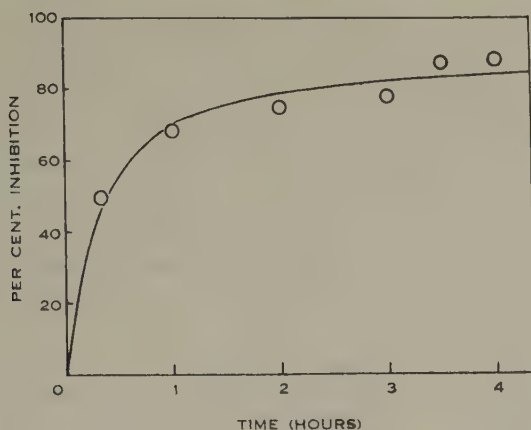


Fig. 1.—Time curve of ninhydrin inhibition of myosin-ATP-ase at pH 9.0. Ninhydrin concentration 0.001M (4.5 μ M per mg. myosin).

and degree of inhibition is illustrated in Figure 2. At pH 9.0 ninhydrin inhibited myosin-ATP-ase at quite high dilutions (90 per cent. at 5 μ M ninhydrin per mg. myosin, or 0.001M ninhydrin, at usual myosin concentrations). Since at a lower pH myosin was more stable and viscosity tests were more conveniently carried out, inhibition of ATP-ase was also studied at pH 7.0. At this pH about seven times the concentration of ninhydrin was needed to produce the same inhibition, as shown in Figure 2.

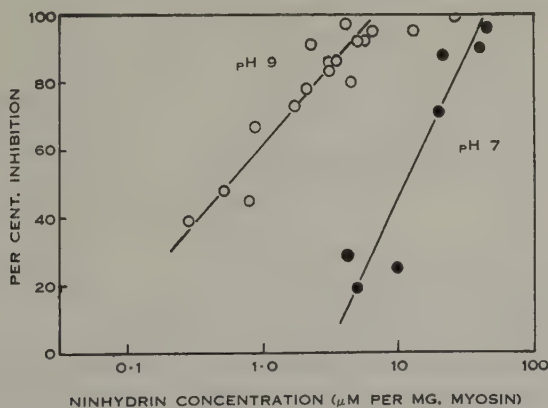


Fig. 2.—Inhibition of myosin-ATP-ase by ninhydrin at pH 7.0 and pH 9.0. All points are derived from experiments in which the ninhydrin had been allowed to act on the enzyme for three or more hours at 5°C.

Cysteine released the inhibition produced by ninhydrin, but complete release was not usually obtained when the ninhydrin had been allowed to act on the myosin for three hours. Figure 3 shows the release of inhibition at pH 9.0 plotted

against relative cysteine concentration, the higher curve being derived from experiments in which cysteine was added after the inhibitor had acted for one hour at 5°C., the lower from experiments in which the inhibitor had acted for three hours at 5°C. Complete release of inhibition after three hours' treatment with ninhydrin was obtained in one experiment when the concentration of cysteine was ten times that of the ninhydrin. The experiments from which Figure 3 is derived were all done with concentrations of ninhydrin of 3.5 μ M per mg. myosin. In two experiments in which ninhydrin at 26 μ M per mg. was allowed to act on myosin for three hours at pH 9.0, cysteine at four times the concentration of the ninhydrin produced only a 25 per cent. release of inhibition.

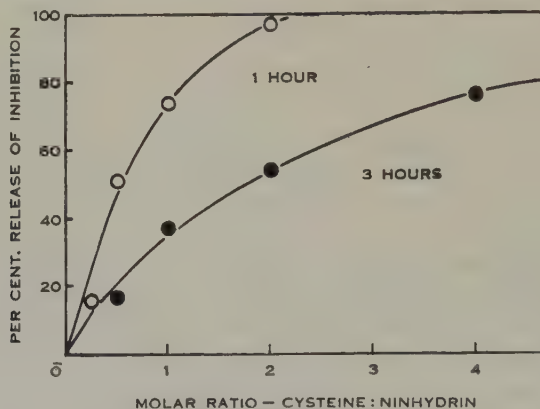


Fig. 3.—Release of ninhydrin inhibition of myosin-ATP-ase at pH 9.0. The upper curve is derived from experiments in which the ninhydrin had been allowed to act on the myosin for one hour at 5°C., the lower from those in which the ninhydrin had been allowed to act for three hours at 5°C. Ninhydrin concentrations in these experiments varied between 3 and 5 μ M/mg. myosin, producing an inhibition of 77-88 per cent. after one hour and 83-92 per cent. after three hours. Each point is derived from at least three individual experiments.

Release of inhibition at pH 7.0, although not studied in as much detail, was apparently quite similar to that illustrated for pH 9.0.

Neither glycine at four times the concentration of the ninhydrin nor alanine at the same or ten times the concentration of the ninhydrin released the inhibition of ATP-ase produced by three hours' treatment by ninhydrin. Thioglycollic acid did release the inhibition, but was less effective than cysteine (50 per cent. release by a concentration four times that of the ninhydrin, after treatment with ninhydrin for three hours at pH 9.0). The addition of alanine at ten times the ninhydrin concentration to either cysteine or thioglycollic acid at four times the ninhydrin concentration did not improve the release of inhibition obtained by either cysteine or thioglycollic acid alone.

IV. EFFECT OF NINHYDRIN ON ACTOMYOSIN FORMATION

In studying the viscosity of myosin solutions it is usual to use concentrations of about 2 mg. of protein per ml. At such high concentrations, however, the treatment of the myosin with ninhydrin at equivalently high concentrations caused profound changes in the physical properties of the protein. The solution became opaque, then set to a stiff gel, which gradually changed to an intense blue colour. With lower absolute concentrations of myosin and ninhydrin, the same relative concentration being maintained, these changes did not occur, and viscosities could be measured readily in the Ostwald apparatus, the viscosity of ninhydrin-treated myosin being very nearly the same as that of untreated myosin.

TABLE 1

EFFECT OF NINHYDRIN ON THE FORMATION AND DISSOCIATION OF ACTOMYOSIN AT pH 7.0*

Myosin Concentration (mg./ml.)	Treatment	Specific Viscosity			
		Myosin Alone	Myosin + Actin	Myosin + Actin + ATP	
0.37	Untreated	1.07	1.28	2 min.	1.09
	Ninhydrin at 45 μ M/mg. myosin†	1.05	1.32	2 min.	1.21
				15 min.	1.12
0.50	Untreated	1.10	1.51	2 min.	1.14
	Ninhydrin at 45 μ M/mg. myosin	1.06	1.64	2 min.	1.43
				15 min.	1.35
0.74	Untreated	1.17	1.83	2 min.	1.26
	Ninhydrin at 45 μ M/mg. myosin	1.23	1.49	‡	

* In all experiments ninhydrin had been allowed to act on the myosin for three or more hours before measurement of the viscosity.

† An enzyme test of this sample revealed 96 per cent. inhibition of the ATP-ase.

‡ Actomyosin precipitated on addition of ATP.

In Tables 1 and 2 are shown the results of viscosity measurements at pH 7.0 and pH 9.0 respectively. It can be seen that the treatment of myosin with ninhydrin in concentrations which produced over 90 per cent. inhibition of the enzyme activity had relatively little effect on the combination of myosin with actin, as demonstrated by the rise in viscosity of myosin solutions on the addition of actin. The relative instability of the ninhydrin-treated actomyosin solutions, however, was demonstrated by their behaviour in the presence of ATP. The addition of ATP to untreated actomyosin resulted in a rapid fall in viscosity to a figure approximating that of the original myosin solution. The addition of ATP to ninhydrin-treated actomyosin at pH 9.0, however, caused the precipitation of the actomyosin. In fact, at the highest concentration recorded, 0.70 mg. per ml., the actomyosin precipitated soon after the addition of actin to the

myosin, before the ATP could be added.* At pH 7.0 and the lower myosin concentrations, the addition of ATP to ninhydrin-treated actomyosin resulted in a drop in viscosity, but the fall was a slow one. Even after 15 minutes the viscosity was still falling slowly, and had not yet reached the level attained by the controls at the first reading after the addition of ATP, i.e. at about two minutes.† This is illustrated in Table 1, where the viscosities of ninhydrin-treated actomyosins at 2 and 15 minutes after the addition of ATP are compared with those of control actomyosins at two minutes after the addition of ATP. Finally,

TABLE 2
EFFECT OF NINHYDRIN ON THE FORMATION AND DISSOCIATION OF ACTOMYOSIN AT pH 9.0*

Myosin Concentration (mg./ml.)	Treatment	Specific Viscosity		
		Myosin Alone	Myosin + Actin	Myosin + Actin + ATP
0.35	Untreated	1.07	1.27	1.07
	Ninhydrin at 5.7 μ M/mg. myosin†	1.05	1.20	‡
0.46	Untreated	1.10	1.39	1.12
	Ninhydrin at 5.4 μ M/mg. myosin	1.09	1.28	‡
0.70	Untreated	1.16	1.75	1.21
	Ninhydrin at 5.7 μ M/mg. myosin	1.10	1.57**	—

* In all experiments ninhydrin had been allowed to act on the myosin for three or more hours before measurement of the viscosity.

† An enzyme test of this sample revealed 92 per cent. inhibition of the ATP-ase.

‡ Actomyosin precipitated on addition of ATP.

** Actomyosin precipitated soon after addition of actin.

at the highest myosin concentration studied at pH 7.0, the addition of ATP caused precipitation, as it had done at the higher pH. It is possible that the drop in viscosity recorded on the addition of ATP to ninhydrin-treated actomyosin at the lower concentrations at pH 7.0 is not a measure of the dissociation of myosin and actin, but represents merely an early stage of the process of aggregation which at higher concentrations is manifested as visible precipitation. When precipitation did not occur immediately it was preceded in our experiments by a drop in viscosity.

* Nitrogen estimations on the precipitated material and the supernatant solution indicated that both myosin and actin must have contributed to the formation of the precipitate, a fact which supports the conclusion that the combination to form actomyosin had preceded precipitation.

† This fall in viscosity with successive readings was much greater in magnitude than the slight successive falls in viscosity due to the thixotropic effect.

V. DIRECT ESTIMATION OF -SH GROUPS

Myosin treated with urea at a concentration of 1 mg. per ml. gave a positive reaction with nitroprusside. The nitroprusside colour could be eliminated by previous treatment of the myosin by ninhydrin, the concentration of ninhydrin required being lower at pH 9.0 than at pH 7.0 (e.g. the colour developed with nitroprusside was about half as strong as the control in myosin treated with ninhydrin at $0.07\ \mu\text{M}$ per mg. at pH 9.0 or $1.4\ \mu\text{M}$ per mg. at pH 7.0). Attempts to estimate -SH quantitatively in the presence of ninhydrin (by *o*-iodosobenzoate or ferricyanide titrations) failed. It is possible that either ninhydrin formed an addition compound which could still be oxidized by the reagents employed, or that reduced ninhydrin was itself oxidized.

VI. DISCUSSION

Although quantitative data on the elimination of -SH by ninhydrin have not been obtained, the indirect evidence that a reaction with thiol groups occurs is quite strong. It comprises in the first place the reversal of ninhydrin inhibition of ATP-ase by cysteine and not by other amino acids, and secondly, the elimination of the nitroprusside colour. This conclusion is further supported by the analogy with alloxan and other evidence from the literature cited in the introduction. At the same time, ninhydrin is known to deaminate and decarboxylate amino acids (Ruhemann 1911), and the occurrence of a reaction of this type with myosin may be reflected in the lower effectiveness of cysteine in releasing the inhibition produced by prolonged treatment with ninhydrin or by higher ninhydrin concentrations. If this view is correct, we may conclude that ninhydrin reacts most rapidly and at the lowest concentrations with -SH, this reaction being much more complete at the higher pH, and that the combination with other groups is slower. As might be expected, the secondary inhibition, due presumably to the reaction with amino and carboxyl groups, is irreversible.

Finally, at quite high ninhydrin concentrations ($> 0.02\text{M}$ at pH 9.0), the physical properties of myosin are affected. The formation of a gel* and development of blue colour are macroscopic evidences of changes in the protein molecule. They parallel closely the clotting and colouration of fibrinogen by ninhydrin, described by Chargaff and Bendich (1943) and ascribed by them to the oxidation of aminoacyl groups of the protein.

Although ninhydrin strongly inhibits the ATP-ase activity of myosin, and this inhibition can most reasonably be ascribed to the elimination of the -SH groups, it has little or no effect on the ability of myosin to combine with actin to produce a complex having a high viscosity. The rise in viscosity on the addition of actin to ninhydrin-treated myosin duplicates almost exactly actomyosin formation with untreated myosin, and it would be difficult to believe that the same phenomenon was not involved in both cases. Our results, however, can not easily be reconciled with those of Bailey and Perry. It is to be noted that Bailey and Perry measured only the viscosity of actomyosin solutions before and

* The consistency of this gel was not affected by the addition of ATP.

after the addition of ATP, and based their calculations of inhibition of actomyosin formation on these figures, whereas we measured also the rise in viscosity on the addition of actin to myosin solutions. It is conceivable that myosin and actin may combine through some groups other than the -SH of myosin, but since intact -SH groups are needed for the attachment of ATP, the displacement of actin by ATP would not be effected in the presence of -SH inhibitors. Evidence against this, however, is to be found in the statement by Bailey and Perry that in their experiments the viscosities of untreated and inhibited actomyosins in the presence of ATP did not differ significantly. Mommaerts (1948) has studied the reaction between ATP and actomyosin in detail, and has concluded that the effect of ATP on the physical behaviour of myosin is entirely independent of the ATP-ase activity, although the experiments on which this conclusion is based are rather uncertain. In view of this conflict in the data it seems that the conclusion of Bailey and Perry that "the viscosity decrease of actomyosin is related to -SH groups and to no other factor" is perhaps an over-simplification.

We have already indicated that the fall in viscosity on the addition of ATP to more dilute solutions of ninhydrin-treated actomyosin at pH 7.0 is probably not an indication of dissociation, but rather of the early stages of precipitation. ATP normally causes "super-precipitation" of actomyosin at KCl concentrations between 0.02M and 0.16M at pH 7.0 (Szent-Györgyi 1947), so that a ninhydrin-treated actomyosin in 0.5M KCl is behaving similarly to an untreated actomyosin at lower KCl concentrations, which may indicate that treatment with ninhydrin has reduced the K^+ combining power of the myosin. Since actin also reduces the K^+ combining power of myosin, precipitation following the addition of actin (Table 2) could also be explained in this way.

VII. ACKNOWLEDGMENTS

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THE DEVELOPMENT OF TYLOSES AND SECRETION OF GUM IN HEARTWOOD FORMATION

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(PLATES 1-7)

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Summary

The origin of both the tyloses and the gum† which are commonly observed in the heartwood vessels of numerous species has been traced to the ray cells.

Tyloses are developed in the heartwood of species in which the width of the aperture of the pits from vessels to ray cells exceeds approximately 10 μ ; in woods in which the width of the pit apertures is less than approximately 10 μ gum is secreted into the heartwood vessels.

There is thus considerable evidence that the activity of the ray cells is a very important factor in the blockage of vessels at heartwood formation, and it is suggested that one of the clues to the development of heartwood may lie in the length of life of the ray cells.

I. INTRODUCTION

Gerry (1914) summarized the literature on the occurrence of tyloses, and this and her own observations showed that although tyloses have in the past been considered one of the characteristics of heartwood, they are in fact quite common in the sapwood of many trees, occurring even in the outermost rings near the bark. Gerry found tyloses in the sapwood of all species in which they occurred in the heartwood. Furthermore, Klein (1923) showed that tyloses could be produced artificially in sapwood by allowing air to enter the vessels following an injury, and he concluded that the formation of tyloses in heartwood was due to the same cause, namely, the contiguity of an air-filled vessel to a living cell of the wood. It has been found that injury to the sapwood of mountain ash (*Eucalyptus regnans* F.v.M.) causes tyloses to form above and below the injury, and that, as in the experiments of Klein, the tylosed area corresponds closely with the area of damage, and consequently with air-filled vessels. In *Acacia* spp. the gum which blocks the vessels both in the heartwood and around an injured area appears to be excreted into the vessels under the same conditions as those which cause tyloses to form in *Eucalyptus regnans*.

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† It is recognized that the secretions into the vessels of the heartwood are of a very varied nature, and that they may be gums, resins, or kinos. This paper is not concerned with the chemical composition of the secreted substances, but only with the details of their secretion, and the term "gum" will therefore be used throughout the article to cover all forms of secretion into heartwood vessels.

These conditions may afford some clue to the factors which cause the change from sapwood to heartwood, and will be the subject of further investigations.

Little work appears to have been done on the actual formation of tyloses or on the secretion of gum. The literature on the subject of both tyloses and gum in wood seems mainly concerned with two aspects, the opposition offered by tyloses to the penetration of preservatives, and the chemical nature of gums and resins. The occurrence of tyloses in heartwood of some species and of gum in that of others seems to have received little attention. The present survey has been undertaken as a result of observations on tyloses in many different woods. These have cast doubt on the common statement that tyloses are a product of ray or parenchyma cells. This may be true as regards the tyloses which form in wholly parenchymatous parts of the plant, such as the leaves (for tyloses may occlude intercellular spaces as well as vessels), but it is not true with regard to their occurrence in wood. During this survey the correlation of vessel-ray pit size with tylosis formation became very apparent, and it is surprising to find only one reference to it in literature. H. von Alten (1909) states that "we were able to show a relationship between the form of the vessel walls and the formation of tyloses, and this seemed to depend especially on the size of the pits." As will be shown, the important feature is not the pitting of the vessel wall in general; it is the size of the pit aperture between vessels and ray cells which determines whether tyloses can form or not.

II. EXPERIMENTAL RESULTS

(a) *Correlation between the Size and Type of Vessel-ray Pitting and the Occurrence of Tyloses*

A survey has been made of the occurrence of tyloses in the wood of over 1,100 genera, using both the material at the Division of Forest Products and the descriptions of woods published by about 47 other wood anatomists. The result of this survey shows that, given the necessary physiological conditions, the presence of tyloses depends on the anatomical structure of the wood itself, and that while tyloses occur almost universally in woods with large vessel-ray pitting (Plate 1, Fig. 1), in which the apertures are large and the borders narrow or insignificant, they are rarely observed in woods with smaller bordered pits (Plate 1, Fig. 2) and never in those in which the vessel-ray pitting is very small or minute. In woods with small bordered vessel-ray pits the occlusion of the vessels in the heartwood is brought about by the secretion of the gum.

Table 1 shows the families in which tyloses occur regularly in all or most of the genera, and Table 2 those in which tyloses have never or very rarely been observed. The measurements have been taken from the available descriptions of woods and checked and added to by measurements made on material at the Division of Forest Products. The closeness of the correlation is immediately apparent. Table 3 shows the families in which tylosis formation occurs in some subfamilies and not in others; here, too, the occurrence of tyloses and

the type and size of vessel-ray pits show the same close correlation. In families in which there is no clear correlation with the botanical subdivision, the pit size is marked for individual species.

TABLE 1

CORRELATION OF OCCURRENCE OF TYLOSES WITH SIZE AND TYPE OF VESSEL-RAY PITTING (FAMILIES WITH TYLOSES*)

Family	Approx. Pit Size (μ)	Pit Type	Family	Approx. Pit Size (μ)	Pit Type
AKANIACEAE	Up to 40	Simple†	LAURACEAE	12-80	Simple
ANACARDIACEAE	16-40	"	LECYTHIDACEAE	Up to 20	"
ARALIACEAE	10-35	"	LYTHRACEAE	15	"
BURSERACEAE	Up to 23	"	MAGNOLIACEAE	Up to 50	"
CAPRIFOLIACEAE	" " 15	"	MONIMIACEAE	" " 30	"
CARYOCARACEAE	Large‡	"	MORACEAE	" " 24	"
COCHLOSPERMACEAE	Up to 16	"	MYRISTICACEAE	" " 45	"
CORNACEAE	15-45	"	OLACACEAE	" " 40	Wide apertures usually simple
CUNONIACEAE	Up to 50	"			
DATISCEAE	" " 50	"			
DIPTEROCARPACEAE	" " 25	"			
ELAEOCARPACEAE	" " 25	"	OLEACEAE	5-10	Wide apertures
ELEAGNACEAE	" " 12	Wide apertures occasionally simple	PLATANACEAE	Up to 12	Simple
			RHIZOPHORACEAE	" " 65	"
			SALICACEAE	12-15	"
EUCRYPHIACEAE	" " 32	Simple	SAPOTACEAE	Up to 30	"
FAGACEAE	17-20	"	SCROPHULARIACEAE	" " 12	"
HAMAMELIDACEAE	20-70	"	SONNERATIACEAE	" " 20	"
HERNANDIACEAE	20	"	THEACEAE	" " 40	"
JUGLANDACEAE	8-15	Wide apertures often simple	ULMACEAE	12-18	"
			URTICACEAE	15-30	"
			VITACEAE	Up to 25	"
JULIANACEAE	Large‡	Simple	VOCHYSIACEAE	10-12	Wide apertures occasionally simple

* Tyloses recorded for all or most of the genera; their absence from records may mean that no mature wood was available for examination.

† "Simple" is used to denote pits in which the border is almost absent; a very narrow border is usually present in some part of the pit.

‡ Recorded, no measurements available.

It will be seen from these tables that there is an overlap between the largest pits in which tyloses do not occur and the smallest in which they do, and that this lies in the region of 8-10 μ . In the Leguminosae (Mimosaceae, Caesalpinaceae, and Papilionaceae) generally, tyloses are absent except from

one subgroup of the Papilionaceae – the Galegeae. It is to this subgroup that *Robina* L., the most tylosed member of the family, with pits up to 16 μ , belongs. Record (1943) has noted tyloses in *Lennea* Klotzsch, *Hebestigma* Urb., and *Gliricidia* H.B. et K. No material was available at the Division of Forest

TABLE 2
CORRELATION OF OCCURRENCE OF TYLOSES WITH SIZE AND TYPE OF VESSEL-RAY FITTING
(FAMILIES WITHOUT TYLOSES*)

Family	Approx. Pit Size (μ)	Pit Type	Family	Approx. Pit Size (μ)	Pit Type
ACERACEAE	8-10	Bordered	MORINGACEAE	8	Bordered
ANONACEAE	4	"	MYOPORACEAE	3-4	"
APOCYNACEAE	4-9	"	MYRICACEAE	8	"
BALANOPSIDACEAE	4	"	NYSSACEAE	5-7	"
BERBERIDACEAE	4	"	OCHNACEAE	2-6	"
BUXACEAE	3	"	OLINIACEAE	3	"
CAPPARIDACEAE	5	"	OPILIACEAE	V. Small	"
CASUARINACEAE	4	"	PITTOSPORACEAE	8	"
CELASTRACEAE	2-7	"	POLYGALACEAE	8-10	"
COMPOSITAE	3-8	"	PROTEACEAE	5-6	"
CRYPTERONIACEAE	6-7	"	RHAMNACEAE	4-8	"
EBENACEAE	3-4	"	RUBIACEAE	3-8	"
EPACRIDACEAE	2-4	"	RUTACEAE	2-5	"
GONYSTYLACEAE	3	"	SABIACEAE	8-9	"
GOODENIACEAE	6-8	"	SAPINDACEAE	3-7	"
HIPPOCASTANACEAE	6	"	STYRACACEAE	5-8	"
HIPPOCRATACEAE	3	"	TAMARICACEAE	3	"
MALVACEAE	8-10	Bordered often with wide apertures	THYMELIACEAE	5-7	"
			ZYGOPHYLLACEAE	2-4	"
MELIACEAE	2-8	Bordered			
MIMOSACEAE	4-8	"			

* Isolated records of tyloses in occasional specimens or by one of several observers have been disregarded.

Products for measurement of pit size in these genera, nor does Record give them, but it is significant that they all belong to the subfamily Galegeae. Tyloses have occasionally been recorded for other genera of the Leguminosae, but such sporadic tyloses may be traumatic, or may occur in woods with pits of the borderline size. As a whole, the Leguminosae, with the exceptions noted, have the heartwood vessels plugged with gum and have small bordered pits, only occasionally reaching 10-12 μ . The tyloses recorded by other observers in occasional species almost invariably occur in woods with pits of the larger size in which there are occasionally vessel-ray pits with unusually narrow borders and wide apertures.

Gerry (1914) has mentioned and figured gum droplets which simulate tyloses, and care has to be taken, in examining slides for tyloses, not to be deceived by artefacts which may resemble tyloses so closely as to mislead even an experienced observer. The only way to be certain in such cases is to use solvents which remove gums but leave the walls of true tyloses intact; such an

TABLE 3

CORRELATION OF OCCURRENCE OF TYLOSES WITH SIZE AND TYPE OF VESSEL-RAY PITTING
(FAMILIES WITH BOTH TYLOSED AND UNTYLOSED GENERA)

Family	Tyloses		No Tyloses	
	Subfamily or Genus	Pit Size and Type	Subfamily or Genus	Pit Size and Type
BETULACEAE	Coryleae	9-10 μ simple†	Betuleae	3-7 μ bordered
BOMBACACEAE	12 genera	Up to 25 μ simple†	6 genera	3-6 μ „
CAESALPINIACEAE	Reported in 3 genera	Up to 12 μ coalescent apertures	43 genera	4-8 μ „
ERICACEAE	Andromedeae	Up to 16 μ simple†	Other sub-families	5-7 μ „
EUPHORBIACEAE*	36 genera	Up to 60 μ simple†	7 genera	4-6 μ „
GUTTIFERAE*	9 genera	Up to 20 μ often simple†	2 genera	6-8 μ „
ICACINACEAE*	2 genera	Up to 30 μ simple†	5 genera	5-8 μ „
LOGANIACEAE*	<i>Fagraea</i>	Up to 25 μ often simple†	Other genera	4-5 μ „
MYRTACEAE*	11 genera	Up to 30 μ simple†	9 genera	3-4 μ „
PAPILIONACEAE	Galegeae	Up to 16 μ wide apertures	Other sub-families	6-10 μ „
ROSACEAE	Chryso-balanoideae	Up to 30 μ simple†	Other sub-families	3-6 μ „
STERCULIACEAE*	4 genera	Up to 50 μ often simple†	13 genera	4-8 μ „
TILIACEAE*	5 genera	Up to 16 μ often simple†	4 genera	3-6 μ „
VERBENACEAE	Viticeae	Up to 15 μ often simple†	Other sub-families	2-7 μ „

* No correlation with division into subfamilies.

† "Simple" is used to denote pits in which the border is almost absent; a very narrow border is usually present in some part of the pit.

artefact is shown in Plate 1, Figure 4, in *Eremophila Mitchelli* Benth. After treatment with 1 per cent. sodium hydroxide the "tyloses" in this wood disappeared. The bordered vessel-ray pits measuring only 3-4 μ in diameter gave the clue, but the deception was absolute on superficial examination.

(b) The Formation of Tyloses or Secretion of Gum by the Ray Cells

Examination of a large number of sections has shown that in every wood where early stages of tylosis formation or gum secretion could be observed, both tyloses and gum came from the ray cells, even when the vessels were also bordered by parenchyma. In all, many hundreds of budding tyloses and early stages in gum secretion were examined. In only three woods were four or five examples found in which tyloses emerged definitely and unmistakably from parenchyma cells (Plate 4, Fig. 1) and only one in which the secretion of gum could similarly be undoubtedly traced to the parenchyma.

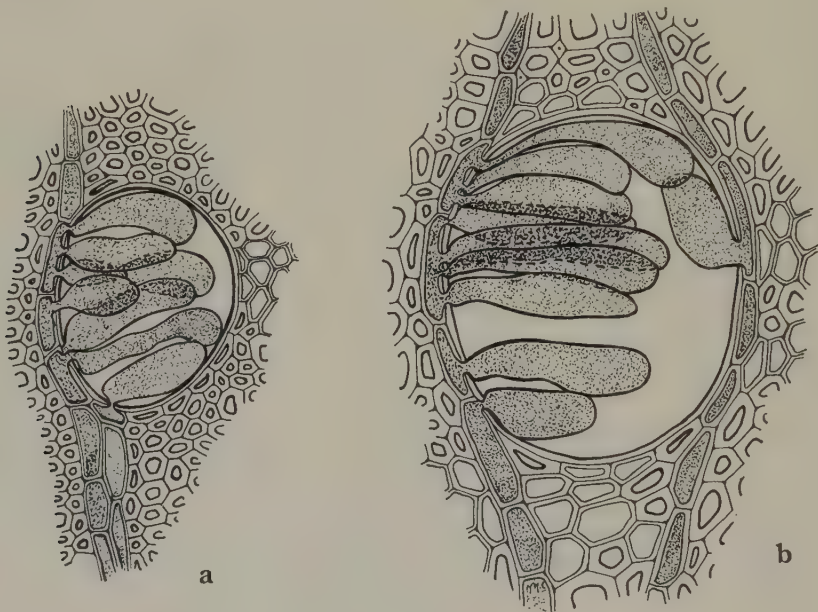


Fig. 1.—Development of tyloses from ray cells.

a. *Hopea ferruginea* Parijs. x approx. 155.

b. *Quercus sundaica* Blume. x approx. 170.

Plate 2, Figures 1-4, and Plate 3, Figures 1 and 2, show that even when the vessels are surrounded by a parenchymatous sheath and are contiguous to a ray for only part of their circumference, the tyloses emerge from the ray cells and not from the parenchyma. In these Plates, early stages in the development of tyloses from the ray cells of *Eucalyptus Dalrympleana* Maiden, *Dipterocarpus retusus* Blume, and *Quercus pseudo-molucca* Blume are shown. In these species of *Eucalyptus* and *Quercus*, the vessels are for the most part bordered with fibres and tracheids, and the tylosis formation from the rays alone might be attributed to the sparsity of parenchyma cells, but this is not the case in *Dipterocarpus retusus*, where the vessels are often bordered almost entirely by rays and parenchyma, and yet the tyloses still arise only from the ray cells. Figure 1 shows the same tissue distribution in *Hopea ferruginea* Parijs and

Quercus undaica Blume, in which the origin of the tyloses is from the rays, although many parenchyma cells border the vessel. Plate 4, Figure 2, shows part of a vessel in *E. miniata* A. Cunn. in which young tyloses arise from a pair of superposed rays.

When vessels which are completely blocked throughout their length by tyloses are examined, it may at first seem impossible that all the tyloses have come from the rays. Further examination of tangential sections shows, however, that the centre of tylosis formation, where the burst pits, can be seen, is always in the rays. Figure 2 (*a-f*), which has been reconstructed from actual photographs of various Dipterocarpaceae, shows how the growth and proliferation of the tyloses has caused them to spread from the immediate neighbourhood of their parent ray and fill the whole of the intervening space between that ray and rays above and below. In (*a*) a single tylosis from a cell of a uniseriate ray has met with no obstacle except the opposite wall of the vessel, and has grown to enormous size, doubling back on itself and effectively blocking the vessel cavity; (*b*) shows a uniseriate ray from every cell of which tyloses have emerged, extending above and below the ray for a considerable distance; (*c*), (*d*), and (*e*) show further examples of the extent to which tyloses from a single ray can proliferate and fill the vessel cavity, while (*f*) shows a vessel similarly blocked, but the tyloses have grown from the cells of four different rays. From these diagrams it will be seen that even when the vessel is flanked by parenchyma, the tyloses arise from the ray cells alone. In Figure 1 (*a* and *b*), cross-sections of earlier stages of similar development are shown.

In woods with small bordered pits between the vessels and the ray cells, in which the secretion of gum replaces the development of tyloses at heartwood formation, the gum may completely fill the cavity of the vessel and extend far above the ray from which it has been secreted; and, as in the case of the tyloses, it is only by examining the very early stages at the junction of sapwood and heartwood that the origin of this gum can be accurately traced. Occasionally, however, when the secretion of gum has gone on for some time, the fresh gum is of a different colour from that previously secreted and the origin from the ray cells is clearly shown. Plate 5, Figure 4, shows a tangential section of *Swartzia tomentosa* D.C., in which the sapwood vessels contained some dark amorphous deposits; on heartwood formation the gum secreted by the ray cells has pushed aside the deposits, giving a characteristic pattern of gum droplets down the vessel wall.

In Plate 6, Figure 1, a vessel of *Albizzia toona* F.M. Bail. is passing through one of the wide parenchyma bands which characterize that wood. The gum, shown enlarged in Plate 6, Figure 2, arises from the ray and not the parenchyma cells; similar examples of early stages in gum formation are shown in Plate 3, Figure 5, and Plate 4, Figures 3-7. In only one example found during an examination of hundreds of slides could the gum be traced with certainty to the parenchyma.

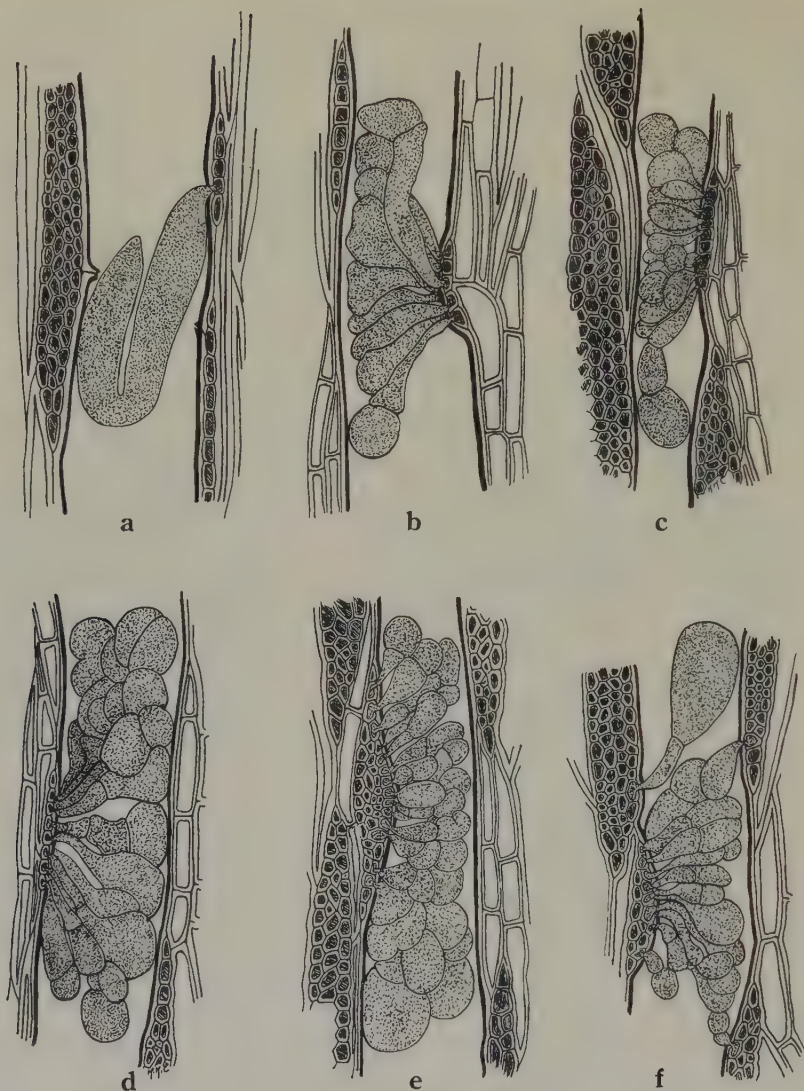


Fig. 2.—Growth and proliferation of tyloses.

- a. *Shorea* sp. Large single tylosis filling the vessel cavity. x approx. 110.
- b. *Hopea ferruginea* Parijs. Tyloses arising from every cell of a ray, growing and filling the whole vessel cavity. x approx. 110.
- c. *Anisoptera marginata* Korth. Tyloses arising from every cell of a ray, growing and filling the whole vessel cavity. x approx. 60.
- d. *Anisoptera marginata* Korth. Tyloses arising from every cell of a ray, growing and filling the whole vessel cavity. x approx. 60.
- e. *Hopea odorata* Roxb. Tyloses arising from every cell of a ray, growing and filling the whole vessel cavity. x approx. 80.
- f. *Hopea odorata* Roxb. Tyloses arising from four different rays to fill the whole vessel cavity. x approx. 80.

In Plate 6, Figure 3, the gum droplet at "A" may at first sight appear to come from a parenchyma cell, but a closer examination shows that a very small uniseriate ray is present (Plate 6, Fig. 4).

Figure 3 (a-c) is made from photographs of some Proteaceae. In each case, the gum can clearly be seen to have come through pits of the ray cells, and not from the surrounding parenchyma; Plate 4, Figures 3-7, shows similar early stages of gum secretion from uniseriate and multiseriate rays. In some

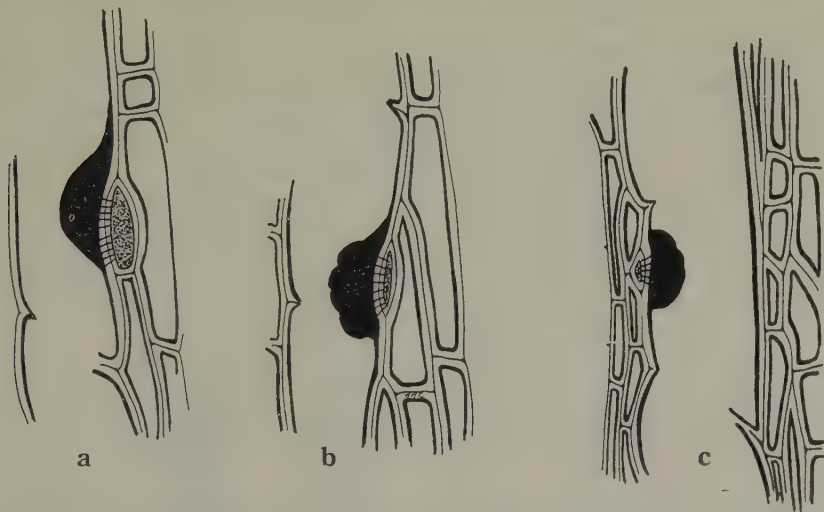


Fig. 3.—Secretion of gum from uniseriate rays which are only one cell high.

a. *Faurea saligna* Harv. \times approx. 250.

b. *Faurea Macnaughtoni* Phillips. \times approx. 250.

c. *Cardwellia sublimis* F.v.M. \times approx. 110.

woods gum and tyloses occur together, generally in the form of gum-filled tyloses (Plate 5, Fig. 3), but occasionally, in addition to gum-filled tyloses, gum may be secreted directly into the vessels. As far as can be seen, the development differs in no way from that in other woods in which only gum or tyloses occur.

III. DISCUSSION

It will be seen from the above that both gum and tyloses in wood appear to have the same origin, namely, the ray cells, and to be formed under similar conditions, i.e. when conduction ceases in the vessels, either as a result of injury or in the normal life of the tree, at the time of heartwood formation.

It was stated by Klein (1923) that tyloses were formed when a living parenchyma cell was in contact with an air-filled vessel, and Gerry (1914) in a résumé of earlier literature on the subject defined them as prolongations of wood or medullary ray parenchyma. These statements should be amended because it has been found that, except in one or two rare instances, both tyloses and gum are formed in the woody parts of trees when a cell of a medullary ray is in contact with a vessel. Both tyloses and gum have been observed to have been

produced from wood parenchyma cells, but such an origin appears to be extremely unusual. From this it would appear that it is the ray cells which play an important and active role in the formation of heartwood, while the wood parenchyma is purely a storage tissue.

In much of the early work on tyloses their origin was attributed to the alteration in pressure in the vessels at the time of truewood formation, allowing the turgid ray or parenchyma cells to push through the pit openings into the vessels. This view is supported by the frequency of tyloses in woods with large simple* vessel-ray pits and the absence of tyloses from woods in which these pits are small and typically bordered. Tyloses appear to be the result of active growth on the part of the ray cells, and to occur not because of, but in spite of, the states of tension and compression that occur in stems. This view accords with those of Winkler (1906) and Klein (1923). The latter reviewed the whole subject and proved to his own satisfaction that tyloses resulted from a vessel which is full of air bordering on a living cell. His experiments were made on injured material, and he considered that he had disproved the tension theory of tylosis formation and proved conclusively that tyloses occurred in his material wherever an air-filled vessel touched upon a living ray or parenchyma cell, and that they did not occur even in injured vessels if these were kept filled with water. On these grounds he denied the existence of a wound stimulus, and suggested that the drying of the pit membrane was sufficient to cause the protrusion and growth of the ray cell.

Gerry's statement that tyloses can occur in the sapwood of all species in which they are present in the heartwood agrees well with Klein's theory. Even in the sapwood occasional vessels may occur in which, through injury or other cause, the sap stream has ceased to function, and in which the conditions postulated by Klein are present.

It does not appear, however, that the work of Klein and Winkler rules out the possibility that an internal stimulus may activate the growth of a ray cell. It is possible that the change from a water-filled to an air-filled vessel may alter the concentration of the growth hormones in the ray cell and thus release an increase of activity within the cell, but it is difficult to imagine that this physical change is of itself sufficient to cause the enormous amount of growth that may be involved in tylosis formation, or the active secretion of large quantities of gum. How great this activity may be is seen from Plate 5, Figure 4, and Plate 7 as well as from Figures 1, 2, and 4. In the formation of a large tylosis such as that shown in Plate 7, the ray cell may have grown to about 40 times its original size, and the secondary wall may have increased from no thickness at all to fill almost the whole of the tylosis.

The growth involved in forming tyloses appears to be exactly similar to that by which secondary thickening of fibres and vessels is laid down. Budding

* The term "simple" is used here to denote pits in which the aperture is very large and the border is almost absent; a border of variable width is usually present in some part of the pit.

tyloses of *Eucalyptus Dalrympleana* Maiden, thin-walled full-grown tyloses of *E. rubida* Deane and Maiden, and sclerosed tyloses of *Gymnacranthera Farquhariana* Warb. were delignified by standard procedure. The budding tyloses of *E. Dalrympleana* and the full-grown tyloses of *E. rubida* are thin-walled, and proved on delignification to consist largely of lignin and to have undergone only very slight secondary thickening. The heartwood of *Gymnacranthera Farquhariana*, on the other hand, contains tylosed vessels in which sometimes almost the whole lumen of the vessel is filled by a "sclerosed tylosis" (Record 1925) in which the wall thickness of the tylosis is many times that of the containing vessel wall. On delignification these tyloses proved to have a wall structure similar to that of adjacent fibres, and to have similar optical and chemical properties. These tyloses are of particular interest as they show clearly the extent of the growth which has resulted from the stimulation of a single ray cell. On account of their very thick walls it is easy to see the limits of a single tylosis both in macerated material and in longitudinal sections (Fig. 4

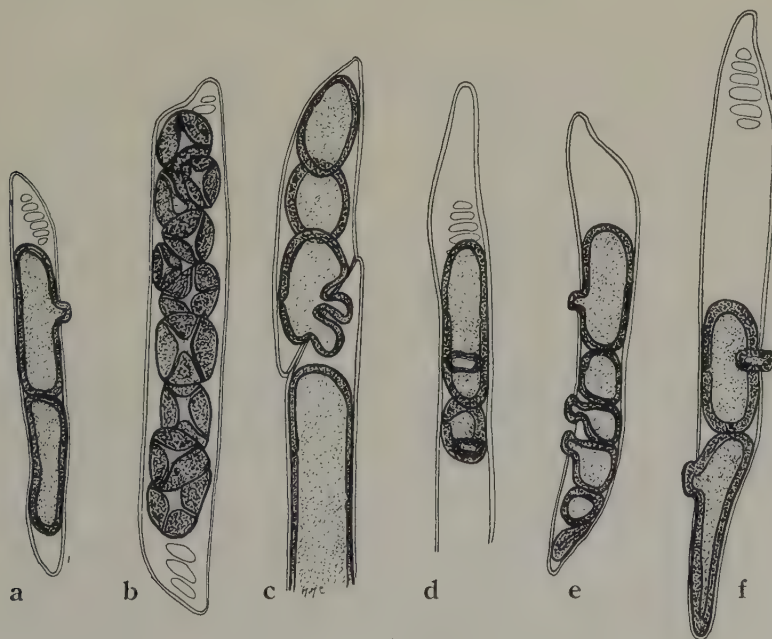


Fig. 4.—Macerated material. Vessel members enclosing sclerosed tyloses. (See text for details.) *Gymnacranthera Farquhariana* Warb. x approx. 72.

and Plate 7). On maceration the vessel members and the tyloses they contain separate from the surrounding tissues (Fig. 4, *a-f*), but, owing to the continuity of the cell wall, the tyloses remain connected with the thickened wall of the ray cell from which they originated. Figure 4 (*d* and *f*) shows these tylosis mother cells in surface view, lying on the vessel member into which the tyloses protrude; (*a*), (*e*), and (*f*) show similar ray cells and their daughter tyloses

as they appear in side view and on tangential sections of the wood (see also Plate 7).

Although the secretion of gum by the ray cells does not involve any actual enlargement of the cell, it must represent a considerable increase of cell activity, resulting not in cell wall formation as in the growth of tyloses, but in the production of an equally large amount of new material in the form of gum.

It is suggested that when the vessels cease to function, whether as the result of injury or on the formation of heartwood, changes of a relatively violent nature occur within the living ray cells, resulting ultimately in the death of the nucleus and complete cessation of all metabolic activity in the cell. But there appears to be a phase before the final dissolution of the nucleus, during which it is stimulated to greater activity. Such activity shows itself in growth, and causes the cell to try to expand. The only part of the cell in which expansion can take place is the unthickened part of the intercellular membrane which stretches across the pits. In woods with large vessel-ray pitting this unthickened membrane may occupy a considerable portion of the part of the ray cell which is contiguous to the vessel, and consequently growth takes place in these areas, forcing the intercellular substance into the vessel, where it forms a tylosis. Further growth may cause the tylosis to proliferate (as in *Dipterocarpus* spp.) or to increase in cell-wall thickness (as in *Gymnacranthera Farquhariana*). In woods with small vessel-ray pitting only a very small portion of the cell wall remains in the unthickened condition, and the consequent resistance of the cell to expansion must be increased. For some reason, not as yet fully investigated, this results in the activity of the cell taking another form, and substances are secreted by the cell into the vessel cavity. On contact with the air in the vessels these substances solidify to form the gum with which vessels of the heartwood or of damaged areas in the sapwood become blocked. Thus the blocking of the vessels by gum and tyloses appears to be a manifestation of the reaction of living ray cells to a stimulus which causes increased activity. Wherever tyloses and gum occur, whether in sapwood, wound wood, or heartwood, they are formed because a *living* cell borders on an air-filled one. Air-filled vessels may exist in the centre of all mature trees, yet not all trees have recognizable heartwood. In past attempts to explain the formation of heartwood the stress has been on the air-filled vessel, and it may be because the problem has always been regarded from this angle that information on it is so meagre. It is possible that a real understanding of the problem of heartwood formation will follow investigations of the living ray cells that occur deep within the tree trunk.

IV. ACKNOWLEDGMENTS

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EXPLANATION OF PLATES 1-7

PLATE 1

- Fig. 1.—*Hedycarya arborea* Forster. Radial longitudinal section showing large vessel-ray pitting. x 225.
 Fig. 2.—*Entandrophragma angolense* Welw. Radial longitudinal section showing small vessel-ray pitting. x 225.
 Fig. 3.—*Eucalyptus Dalrympleana* Maiden. Transverse section of vessel showing budding tyloses. x 470.
 Fig. 4.—*Eremophila Mitchelli* Benth. Tangential longitudinal section of vessel showing oil droplets which may easily be confused with tyloses. x 1000.

PLATE 2

- Fig. 1.—*Eucalyptus Dalrympleana* Maiden. Transverse section showing development of tyloses from ray cells. x 180.
 Fig. 2.—*Eucalyptus Dalrympleana* Maiden. Transverse section showing development of tyloses from ray cells. x 85.
 Fig. 3.—*Dipterocarpus retusus* Blume. Transverse section showing tyloses of various sizes developing from ray cells. x 170.
 Fig. 4.—*Quercus pseudo-molucca* Blume. Transverse section showing young tyloses developing from ray cells. x 170.

PLATE 3

- Fig. 1.—*Eucalyptus Dalrympleana* Maiden. Transverse section showing one-sided development of tyloses where the vessel is contiguous to a ray on one side only. x 160.
 Fig. 2.—*Dipterocarpus retusus* Blume. Transverse section showing young tyloses arising from ray cells although the vessel is also touched by vasicentric parenchyma. x 170.
 Fig. 3.—*Faurea saligna* Harv. Transverse section. Gum secretion on side contiguous to ray cells. x 400.
 Fig. 4.—*Carnarvonnia araliaefolia* F.v.M. Transverse section. Gum secretion on side contiguous to ray cells. x 125.
 Fig. 5.—*Melia Azedarach* Linn. Tangential longitudinal section showing gum secretion from ray cells. x 180.
 Fig. 6.—*Carapa moluccensis* Lamk. Transverse section showing small gum drop and vessel-ray pitting. x 400.

PLATE 4

- Fig. 1.—*Antiaris* sp. Transverse section showing exceptional case of tylosis development from parenchyma. x 500.
 Fig. 2.—*Eucalyptus miniata* A. Cunn. Tangential longitudinal section showing tyloses developing from pair of superposed rays. x 180.
 Fig. 3.—*Melia Azedarach* Linn. Tangential longitudinal section showing secretion of gum from ray cells. x 70.
 Fig. 4.—*Khaya anthotheca* D.C. Tangential longitudinal section showing secretion of gum from ray cells. x 40.
 Fig. 5.—*Carnarvonnia araliaefolia* F.v.M. Tangential longitudinal section showing secretion of gum from cells of multi-seriate ray. x 150.

Fig. 6.—*Carnarvonia araliaefolia* F.v.M. Gum secretion from cells of uniseriate ray. x 150.

Fig. 7.—*Acacia mollissima* Willd. Tangential longitudinal section. Secretion of gum by ray cells. x 225.

PLATE 5

Fig. 1.—*Grevillea robusta* A. Cunn. Transverse section showing gum-filled vessels occurring only along the ray. x 75.

Fig. 2.—*Faurea saligna* Harv. Transverse section showing gum-filled vessels occurring only along the ray. x 75.

Fig. 3.—*Eucalyptus Blakleyi* Maiden. Transverse section showing gum in tyloses. x 700.

Fig. 4.—*Swartzia tomentosa* D.C. Tangential longitudinal section showing secretion of fresh gum into a vessel already filled with black amorphous deposits. x 110.

PLATE 6

Fig. 1.—*Albizzia toona* F.M. Bail. The vessel is surrounded by parenchyma but the gum is secreted by the ray cells. x 43.

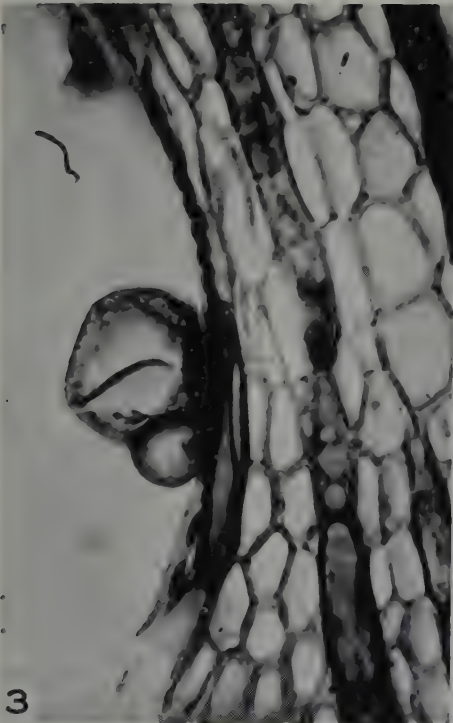
Fig. 2.—*Albizzia toona* F.M. Bail. Part of the above enlarged. x 125.

Fig. 3.—*Melia dubia* Hiern. At "A" the gum is secreted by a small unicellular ray. x 115.

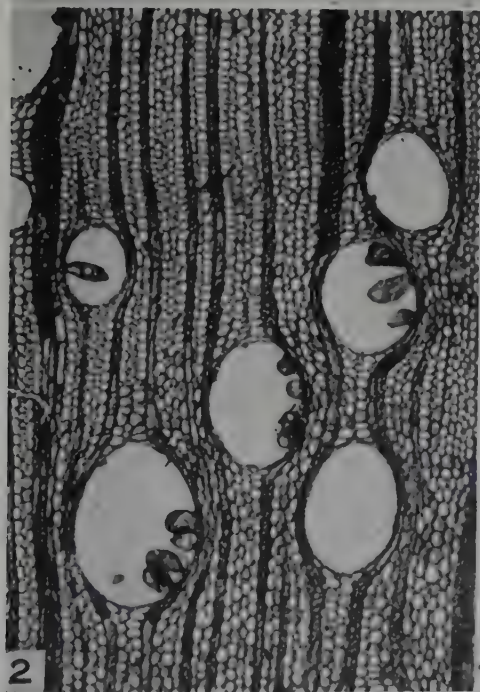
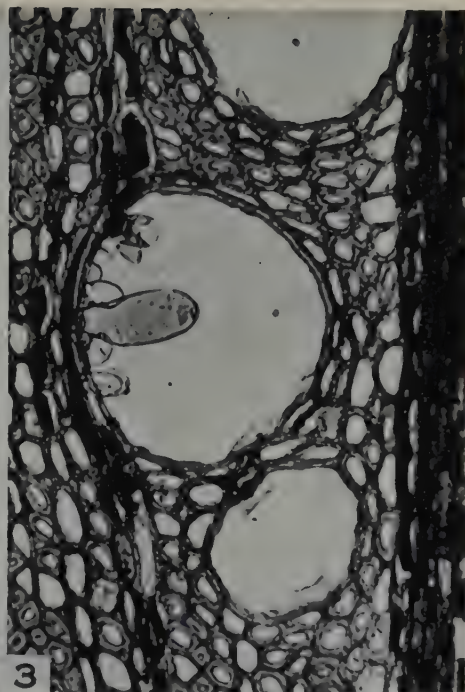
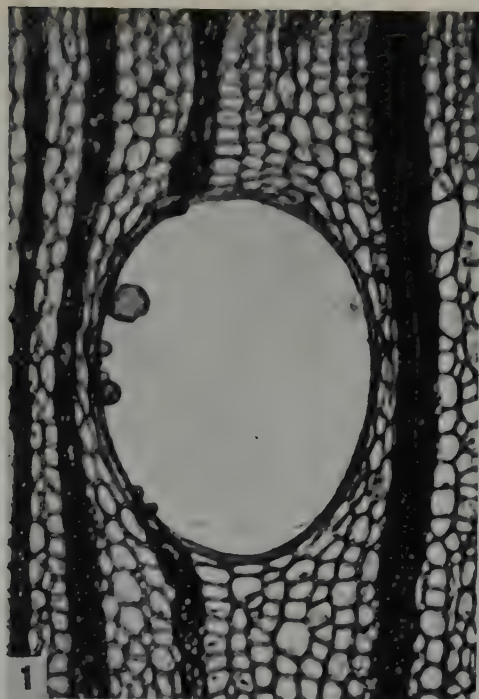
Fig. 4.—*Melia dubia* Hiern. "A" enlarged. x 600.

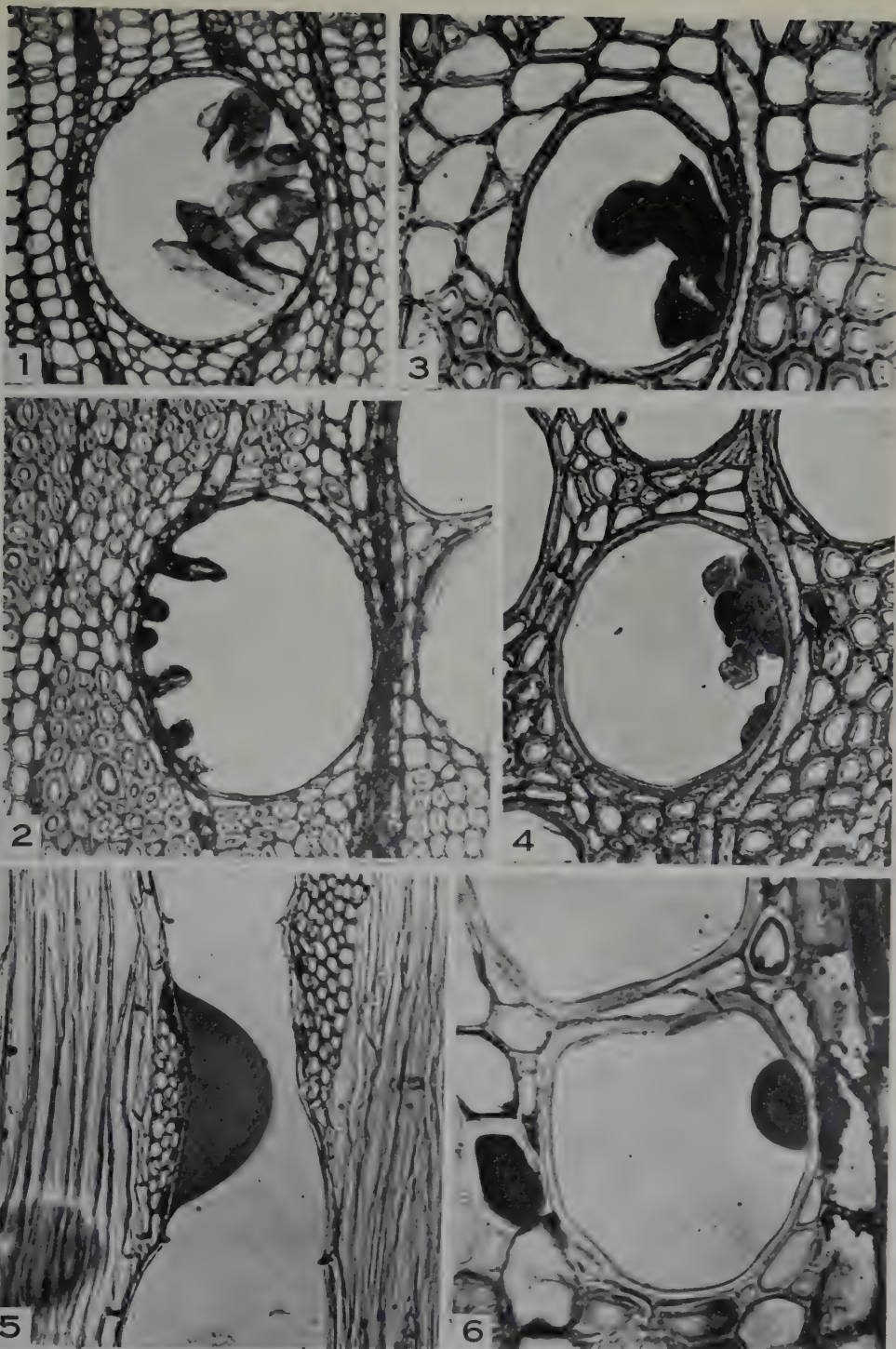
PLATE 7

Gymnacranthera Farquhariana Warb. Tangential longitudinal section of sclerosed tylosis, showing the ray cell from which it originated. x 280.

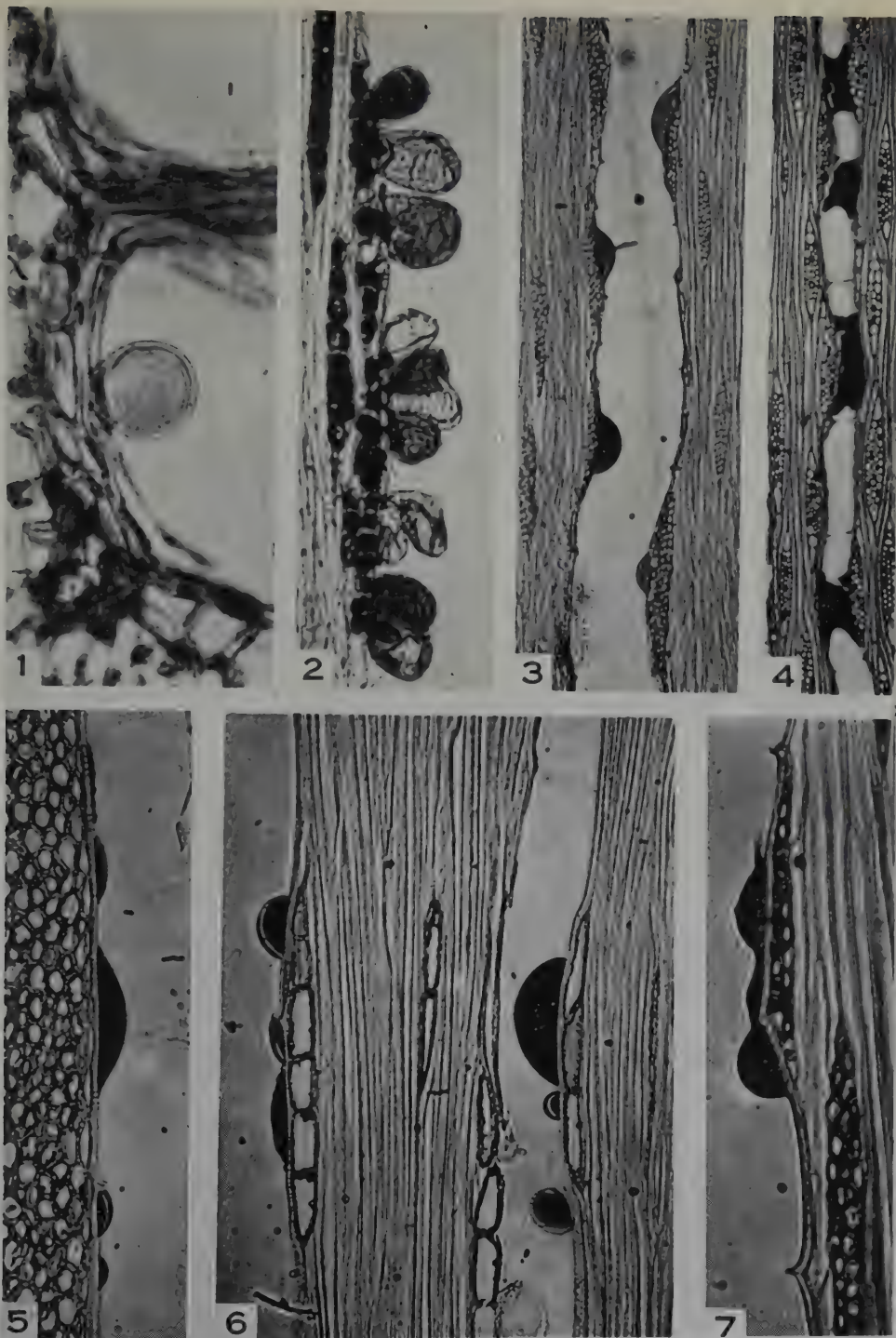


CHATTAWAY.—THE DEVELOPMENT OF TYLOSES AND SECRETION OF GUM IN HEARTWOOD FORMATION

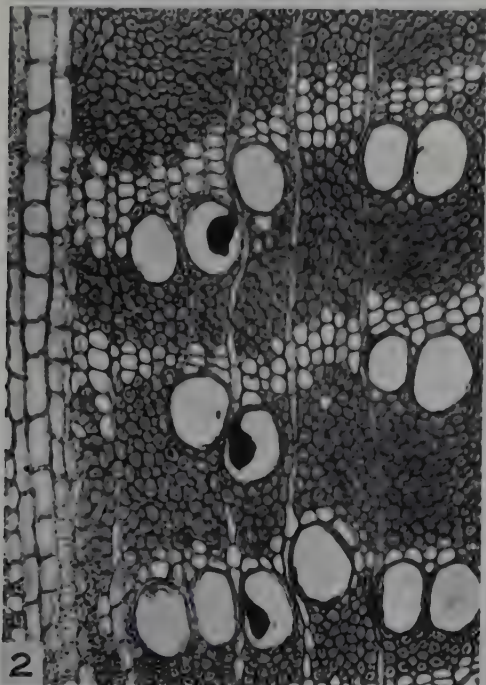
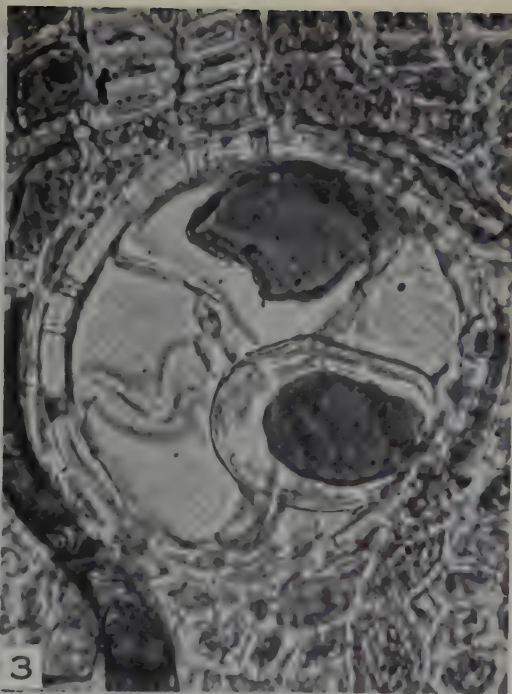
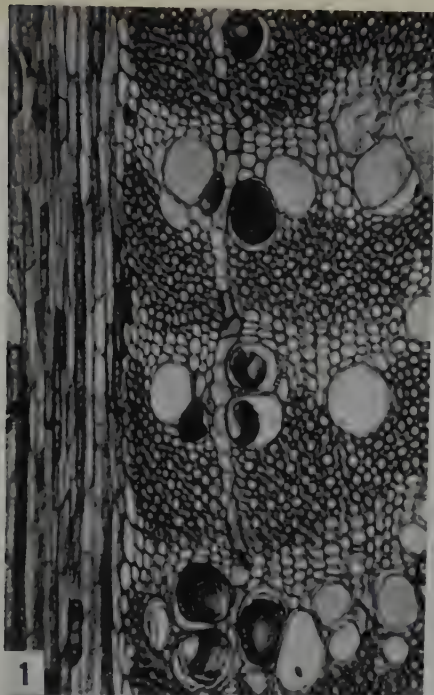




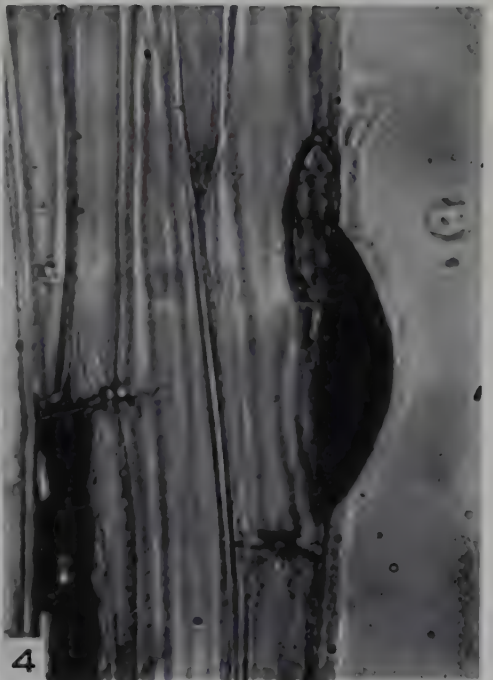
CHATTAWAY.—THE DEVELOPMENT OF TYLOSES AND SECRETION OF GUM IN HEARTWOOD FORMATION



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CHATTAWAY.—THE DEVELOPMENT OF TYLOSES AND SECRETION OF GUM IN HEARTWOOD FORMATION

MICROSPOROGENESIS, MACROSPOROGENESIS, AND DEVELOPMENT OF THE MACROGAMETOPHYTE AND SEEDS OF *DUBOISIA LEICHHARDTII* (F.v.M.) AND *D. MYOPOROIDES* (R.Br.)

By C. BARNARD*

[Manuscript received April 6, 1949]

Summary

In *Duboisia Leichhardtii* and *D. myoporoides* macrosporangogenesis and the development of the embryo-sac are similar to the descriptions reported for other genera of the family Solanaceae.

The haploid number of chromosomes in both species is 30.

A generative and vegetative nucleus is formed in each microspore which later becomes filled with starch grains and uninucleate as a result of degeneration of the vegetative nucleus. At maturity the pollen grains are devoid of starch and are uninucleate. Division of the generative nucleus to form two male nuclei presumably occurs just prior to the discharge of the pollen tube.

Double fertilization occurs and the fusion of one male nucleus with the fusion nucleus takes place before fertilization of the egg.

Embryogeny is comparable to published descriptions for other genera of the Solanaceae but the development of the endosperm is different.

A primary endosperm of large thin-walled cells is present until the stage when the ovules have almost reached mature seed size and the embryo is at about the 14-celled stage. Meristematic activity commencing in cells in the vicinity of the embryo then results in the formation of a dense compact "secondary" endosperm. Cells of the secondary endosperm become filled with oil.

Degeneration of whole ovules may take place but "empty" seed develop from ovules which contain no embryo and in which secondary endosperm has not formed.

I. INTRODUCTION

During the course of experimental work with *Duboisia myoporoides* and *D. Leichhardtii* considerable difficulty has been experienced in germinating seed. As previously reported (Loftus Hills and Kelenyi 1946) "most seed samples although they appeared normal contained a variable proportion of empty or partly filled seed. The number of full seeds in a series of samples derived from individual trees of both species ranged from nil to 80 per cent. and averaged 13 per cent. The fluctuations did not appear to be related to the geographical origin of the samples or to the year in which the seed was collected. The phenomenon may be due to self sterility, lack of effective pollination or some other cause and an explanation is now being sought."

As part of the enquiry into the reason for the "emptiness" of such a high proportion of *Duboisia* seeds, an examination was made of the morphological development of the ovule and seed of both species. Material was collected

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from young trees in experimental plots at Canberra during 1946-47 and 1948-49, fixed in formalin acetic alcohol, embedded in paraffin wax, and microtomed. Mature seed of *D. Leichhardtii* collected in 1946-47 was 85-90 per cent. sound and only 10-15 per cent. empty. In 1948-49 there was an even lower proportion of empty seed in *D. Leichhardtii* though in *D. myoporoides* almost 80 per cent. was empty.

Germination of the morphologically sound seed in both species was, however, most unsatisfactory and it became apparent that a propagation problem unrelated to seed emptiness existed. Investigations in progress aim to discover the conditions which the sound seed requires for proper germination.

The observations which were made during the course of these studies on the microsporogenesis and the development of the macrogametophyte and the ovule are now presented, because no account of the life history of any of the three species of *Duboisia* has been reported previously.

In most respects development is comparable to that in other genera of the Solanaceae. The main difference is in the development of the endosperm which in *Duboisia* is of a type which so far as the author is aware has not been previously described. The emptiness of seed is probably caused primarily by lack of fertilization. The bulk of the material examined was of *D. Leichhardtii*. This species has therefore been made the main subject of description, observation on *D. myoporoides* being made in a comparative manner.

II. MACROSPOROGENESIS

A single integument is developed in the young ovule and an hypodermal archesporial cell functions directly as the megaspore mother cell. The nucellus consists of a single layer of cells enclosing the megaspore mother cell and projects beyond the integument at this stage (Plate 1, Fig. 1).

The first division of the nucleus of the megaspore mother cell is meiotic and at metaphase the spindle is parallel with the length of the cell (Plate 1, Fig. 2). The integument now slightly projects beyond the nucellus. A cell wall is formed between the two daughter nuclei (Plate 1, Fig. 3) and these cells divide simultaneously to form a linear tetrad of four megaspores. The integument meanwhile continues to grow rapidly and "embeds" the nucellus and by the time the four megaspores have been formed a micropyle of some length exists.

The three megaspores nearest the micropyle soon degenerate whilst the chalazal megaspore increases in size and becomes functional (Plate 1, Figs. 4 and 5). Macrosporogenesis in *Duboisia* is therefore similar to that in other described species of Solanaceae (e.g. Cooper 1931; Rees-Leonard 1935; Smith 1935; Cochran 1938).

III. DEVELOPMENT OF MACROGAMETOPHYTE (EMBRYO-SAC)

The functional megaspore increases in size considerably before the first division of its nucleus and becomes roughly egg-shaped with a narrow projection at the chalazal end. The nucleus divides and one of the daughter nuclei passes

to the micropylar and one to the chalazal end. Each again divides to give two nuclei at the chalazal end and two at the micropylar (Plate 1, Fig. 6). During this time the nucellus degenerates and disappears entirely at the micropylar end though persisting for some time at the chalazal end of the sac. The innermost layer of cells of the integument assumes a palisade-like arrangement. This "tapetum" is just becoming apparent at the stage represented in Plate 1, Figure 6, and is very definite by the time the embryo-sac is mature, as shown in Plate 1, Figure 7.

Development of the embryo-sac proceeds in a normal manner to an 8-nucleate stage. A normal egg apparatus is developed and the two polar nuclei come together usually at the micropylar end of the sac where they fuse. The three antipodal nuclei are most ephemeral and could rarely be found by the time the egg apparatus had been organized and the polar nuclei moved together.

The embryo-sac with the egg and synergids at the micropylar end and the two polar nuclei about to fuse is shown in Plate 1, Figures 7 and 8. These photographs are of successive sections of the same embryo-sac. In Plate 1, Figure 7, the two polar nuclei are shown, while in Plate 1, Figure 8, the egg and two synergids are visible together with the cytoplasm surrounding the polar nuclei just below and to the left of the egg. Not properly visible in the photograph but discernible in the section are two antipodal nuclei at the chalazal end of the sac. The egg apparatus of a mature embryo-sac is shown in Plate 1, Figure 9. It is a diagrammatically normal type. The nucleus of each pear-shaped synergid lies at its narrow end above a vacuole. The egg cell is large with the nucleus lying within the side farthest removed from the micropyle. The large fusion nucleus occupies a position just below and to one side of the egg. In *D. myoporoides* the egg apparatus presents a slightly different appearance in that the large fusion nucleus is more often found placed against the wall of the sac and to one side of the egg rather than below it and the synergids are fuller, not so narrow, and do not stain so darkly.

Development of the embryo-sac is thus of quite normal solanaceous type and in detail approaches very closely to that of *Solanum* as described by Rees-Leonard (1935).

IV. MICROSPOROGENESIS

Differentiation of sporogenous cells in the anther occurs at a very early stage. Development of the anther was not followed in detail but appears to be essentially similar in the early stages to that described for *Capsicum* by Cochran (1938). At the time the pollen mother cells have formed, the wall in the anther consists of an epidermis, an undifferentiated endothecium, one or two layers of darkly-staining tangentially elongated cells (referred to as the middle layer or outer tapetum), and a tapetum of binucleate cells. The cells of the tapetum enlarge and separate as the pollen mother cells separate and become round prior to meiosis. At meiosis the haploid number of chromosomes is observed to be 30 (Plate 3, Fig. 4). The diploid number has been confirmed

at 60 in both species in mitotic root-tip divisions. Meiosis occurs in the microspore mother cells before the megaspore mother cell, and when the latter divides tetrads or free pollen are present in the anthers.

The tapetum commences to disorganize about the time of tetrad division of the microspores. The cells of the middle layer also degenerate and appear to commence to do so before the tapetal cells. By the time the functional megaspore has developed (as in Plate 1, Fig. 1) the tapetum is staining lightly and the endothelial cells elongating radially.

The nucleus of the microspore divides into a generative and vegetative nucleus, the latter being circumscribed by the formation of a cell wall (Plate 3, Fig. 5). The microspores later become packed with starch grains and the vegetative nucleus degenerates (Plate 3, Fig. 6). Disorganization of the tapetum continues and it has disappeared by the time the megaspore is binucleate.

The starch gradually disappears in the pollen grains as they mature and the connective tissue between the locules of the anther breaks down. At anthesis the pollen grains are uninucleate (rarely binucleate), devoid of starch, and have a well-defined intine and exine and three germ pores (Plate 3, Fig. 7), and there is only one sac in the anther.

Upon germination* the generative nucleus may pass into the pollen tube almost immediately or the pollen tube may grow for quite a considerable length whilst the nucleus remains in the grain (Plate 3, Figs. 8 and 9). The division of the generative nucleus into the two male gametes was not observed in artificial culture and presumably does not take place until the pollen tube reaches the embryo-sac.

V. FERTILIZATION

The pollen tube enters the embryo-sac through the micropyle. At the time of entry the tip of the tube is swollen and stains very deeply (Plate 1, Fig. 10). The two male nuclei may sometimes be distinguished before the tip bursts. Even after discharge the deeply-staining contents of the tube considerably obscure one's view. The synergids are disorganized upon the entry of the tube. One male nucleus fuses with the fusion nucleus and the second male nucleus with the egg. The fusion of the male nucleus with the fusion nucleus always occurs first and the endosperm nucleus has passed to the chalazal end of the sac and may have divided before fertilization of the egg occurs. In Plate 1, Figures 11 and 12, photographs of two successive sections show one male nucleus about to fuse with the fusion nucleus whilst the other is just discharging from the pollen tip. For some time after the fusion of the egg and the male nucleus the zygote nucleus may remain binucleolate.

* Germination of pollen grains was effected by dusting on to a flattened drop of 15 per cent. sucrose solution to which 0.5 per cent. agar had been added on a slide and placing in a moist chamber. When germination had taken place the preparation was fixed in Alcohol-Acetic (3 pts. to 1 pt.), cleared in absolute alcohol, stained in 1 per cent. lacmoid in 50 per cent. aqueous glacial acetic, rinsed in absolute alcohol, drained, and a drop of euparal and a cover slip added. In this way very satisfactory permanent preparations could be made.

Fertilization in *Duboisia* differs mostly from the observations made by Cooper (1931) for *Lycopersicum*, by Cochran (1938) for *Capsicum*, and by Clarke (1940) for *Solanum*, in that the fusion of the egg and the second male nucleus is delayed.

VI. DEVELOPMENT OF EMBRYO, ENDOSPERM, AND SEED

After fertilization the endosperm nucleus migrates to the chalazal end of the sac and undergoes a number of divisions before the first division of the zygote. Several densely cytoplasmic endosperm cells "settle" at the bottom of the sac and form a base upon which a "free" nuclear endosperm develops. The zygote becomes pear-shaped and the stalk end fixes the young embryo to the integument at the micropylar end of the sac (Plate 2, Fig. 1).

The development of the embryo appears to be quite normal and follows generally the course described for other solanaceous plants. *Duboisia* also agrees with other accounts in that the first division of the zygote does not take place until some considerable time after fertilization and until after a varying amount of endosperm tissue has been formed. Here, however, agreement ceases and the development of the endosperm in *Duboisia* differs most markedly from other species of this family which have been described. Endosperm development is nothing like that figured by Smith (1935) for the tomato or Cochran (1938) for pepper.

The first formed endosperm cells are densely cytoplasmic — about five or six in number — and form a compact mass at the chalazal end of the sac. Upon this "base" other endosperm cells are developed stretching into the sac and a number of "free" nuclei formed which settle around the periphery of the sac. Prior to the formation of cell walls, there are well-defined cytoplasmic units at the chalazal end of the sac. (These units were called cells above.) Further from the chalazal end the cytoplasmic units become less well defined until in the centre of the sac or at the micropylar end there is the appearance of a number of nuclear units in one cytoplasmic unit, i.e. free nuclei. Because of the very early division into cytoplasmic units at the chalazal end of the sac, it is difficult to say when cell walls are first formed, but they are developed before the first division of the zygote (Plate 2, Fig. 3).

The ovule grows very rapidly by the development of the integument and the cells of the endosperm tissue become larger. The ovule develops to almost double the size shown in Plate 2, Figure 3, before the first division of the zygote. The enlargement of the ovule and the cells of the endosperm continues until at the stage when the embryo is 4-5-celled an appearance in section is presented as shown in Plate 1, Figure 4. The embryo is developed at the micropylar end of the ovule, the centre of which appears as a cavity. Actually this cavity is filled with endosperm tissue which now consists of very large cells with walls of extreme delicacy. The cells of the outer layer of the integument continually enlarge and later develop to form the seed testa.

Plate 2, Figures 5-7, shows the development of the embryo to the stage when about 12-14 cells are seen in longitudinal section. About this stage

meristematic activity recommences in the endosperm cells in the vicinity of the embryo and smaller celled secondary endosperm tissue starts to form (Plate 2, Figs. 7 and 8). This development of a compact endosperm tissue starting at the micropylar end of the sac continues until the whole sac is filled. In Plate 2, Figure 9, an intermediate stage is shown. The embryo is larger than that shown in Plate 2, Figure 8, and the new endosperm tissue fills most of the sac. By the time the embryo has reached the stage shown in Plate 2, Figure 10, the sac is filled with a dense endosperm tissue. Plate 2, Figures 11 and 12, and Plate 3, Figures 1-2, show further stages in development which result in a well-formed curved embryo.

The developing endosperm tissue absorbs the inner tissues of the integument, and as the seed matures its cells become filled with oil. The cells of the outer layer of the integument have continued to enlarge during the growth of the ovule. Their inner and radial walls become very much thickened. Upon maturation of the seed the outer walls break down and the thickened radial walls form the walls of the pits upon the surface.

VII. DEGENERATION OF THE OVULES AND EMPTY SEED

Degeneration of some of the ovules may occur at almost any stage of development but most commonly after the formation of the megaspore mother cell and before maturation of the embryo-sac. The first signs of degeneration occur in the nuclei, then the sac shrinks and stains very deeply. Following the disorganization of the sac the whole ovule degenerates and aborts.

Those ovules which develop into mature but "empty" seeds never contain an embryo nor any secondary endosperm tissue. However, primary endosperm tissue is formed and this tissue is composed of smaller and not such thin walled cells as when an embryo has been formed. In Plate 3, Figure 3, a longitudinal section through an almost mature empty seed is shown. The endosperm tissue is practically devoid of any stored metabolites, and when the seed fully matures and dries, this tissue breaks down leaving a large cavity within the testa.

It is improbable that in the development of such seed the egg was ever fertilized and that the lack of an embryo is due to degeneration. No instances of embryonal degeneration have been observed. It could not be determined whether fertilization of the fusion nucleus occurs and endosperm formation is normal or whether the fusion nucleus develops to form the endosperm tissue without fertilization. In view of the rather different nature of the tissue of the primary endosperm ovules without embryos it would seem that the latter alternative is the most probable. Secondary endosperm does not develop and it may be taken that the presence of an embryo is necessary to stimulate the formation of this tissue.

VIII. ACKNOWLEDGMENT

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EXPLANATION OF PLATES 1-3

Photomicrographs of *Duboisia*.

PLATE 1

- Fig. 1.—Median L.S. young ovule showing megaspore mother cell at "M." x 276.
- Fig. 2.—L.S. ovule with megaspore mother cell showing early telophase of first (meiotic) division. x 517.
- Fig. 3.—L.S. ovule with megaspore mother cell in late telophase of first division showing deposition of dividing wall. x 564.
- Fig. 4.—L.S. ovule. Three micropylar megaspores have degenerated. The chalazal megaspore shows more clearly in the next section. x 238.
- Fig. 5.—L.S. ovule showing functional megaspore and two of the three degenerated micropylar megaspores. x 255.
- Fig. 6.—L.S. ovule showing developing megaspore at the 4-nucleate stage. Two nuclei are visible at the chalazal end but only one appears in this section at the micropylar end of the megaspore. x 255.
- Fig. 7.—L.S. embryo-sac showing the two polar nuclei about to fuse. They are situated at the micropylar end of the sac. x 276.
- Fig. 8.—L.S. embryo-sac. This section is next in the series to that shown in Figure 7. The two synergids and egg at "E" can be seen. Just to the right of the egg the surrounding cytoplasm of the polar nuclei is visible. x 276.
- Fig. 9.—L.S. micropylar end of embryo-sac showing mature egg apparatus in detail. The fusion nucleus at "F" and the egg at "E." There is a large and conspicuous vacuole below the nucleus of each synergid (S). x 429.
- Fig. 10.—L.S. of micropylar end of embryo-sac showing entry of pollen tube at "P." Egg nucleus at "E." Fusion nucleus at "F." x 429.
- Figs. 11 and 12.—L.S. micropylar end of embryo-sac after discharge of pollen tube. These two figures are photographs of successive sections. In Figure 11 the egg nucleus is at "E" and one male nucleus at "M." Just below the male nucleus is the edge of the fusion nucleus. In Figure 12 the fusion nucleus (F) is cut medianly and the second male nucleus is at "M." Pollen tube at "P." x 429.

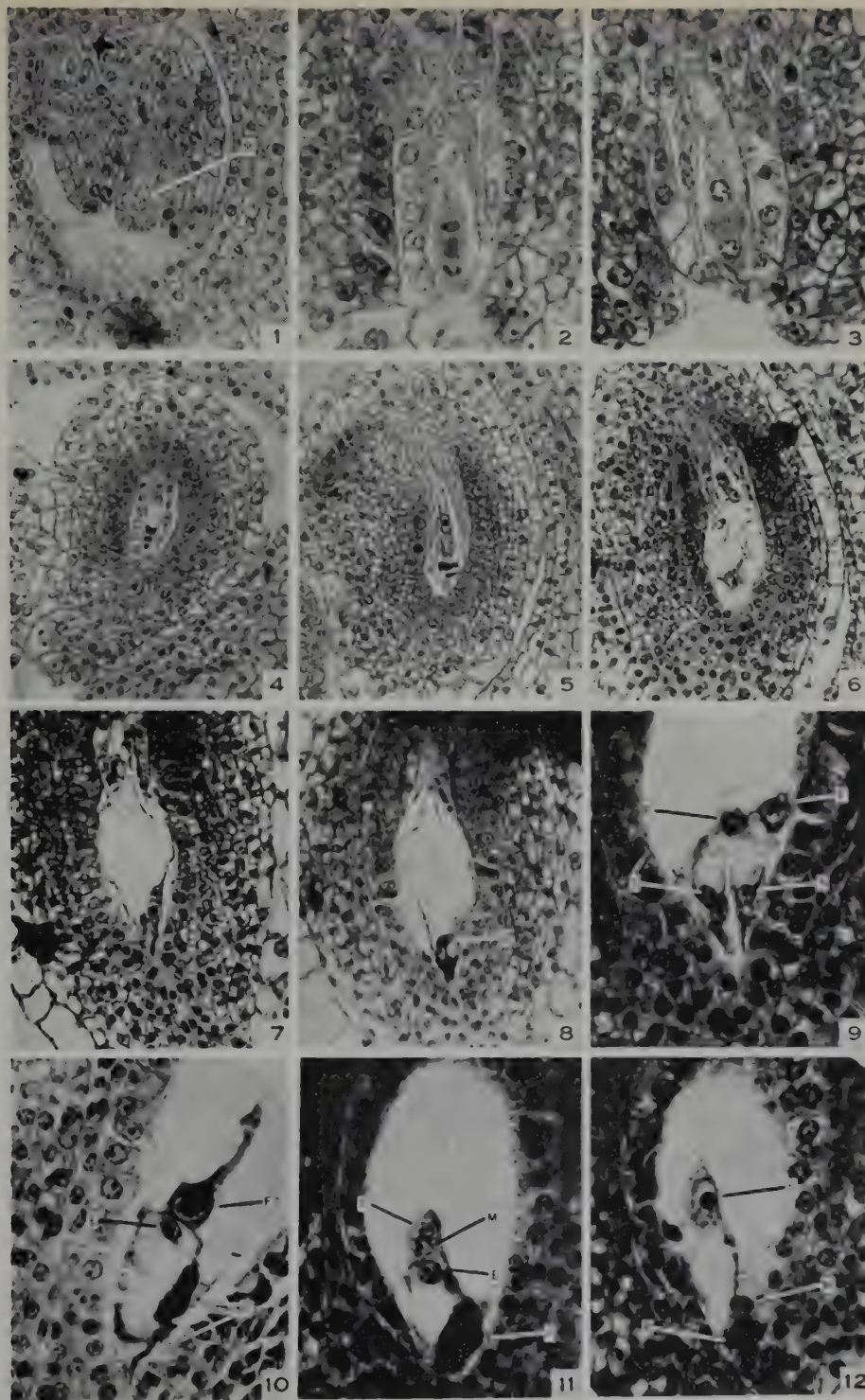
PLATE 2

- Fig. 1.—L.S. embryo-sac after fertilization. Single celled embryo at "EM" and the two synergids. The endosperm nucleus has migrated to chalazal end of sac and divided (EN). x 108.
- Fig. 2.—L.S. embryo-sac showing single celled embryo at "EM" and developing endosperm at "EN." x 163.

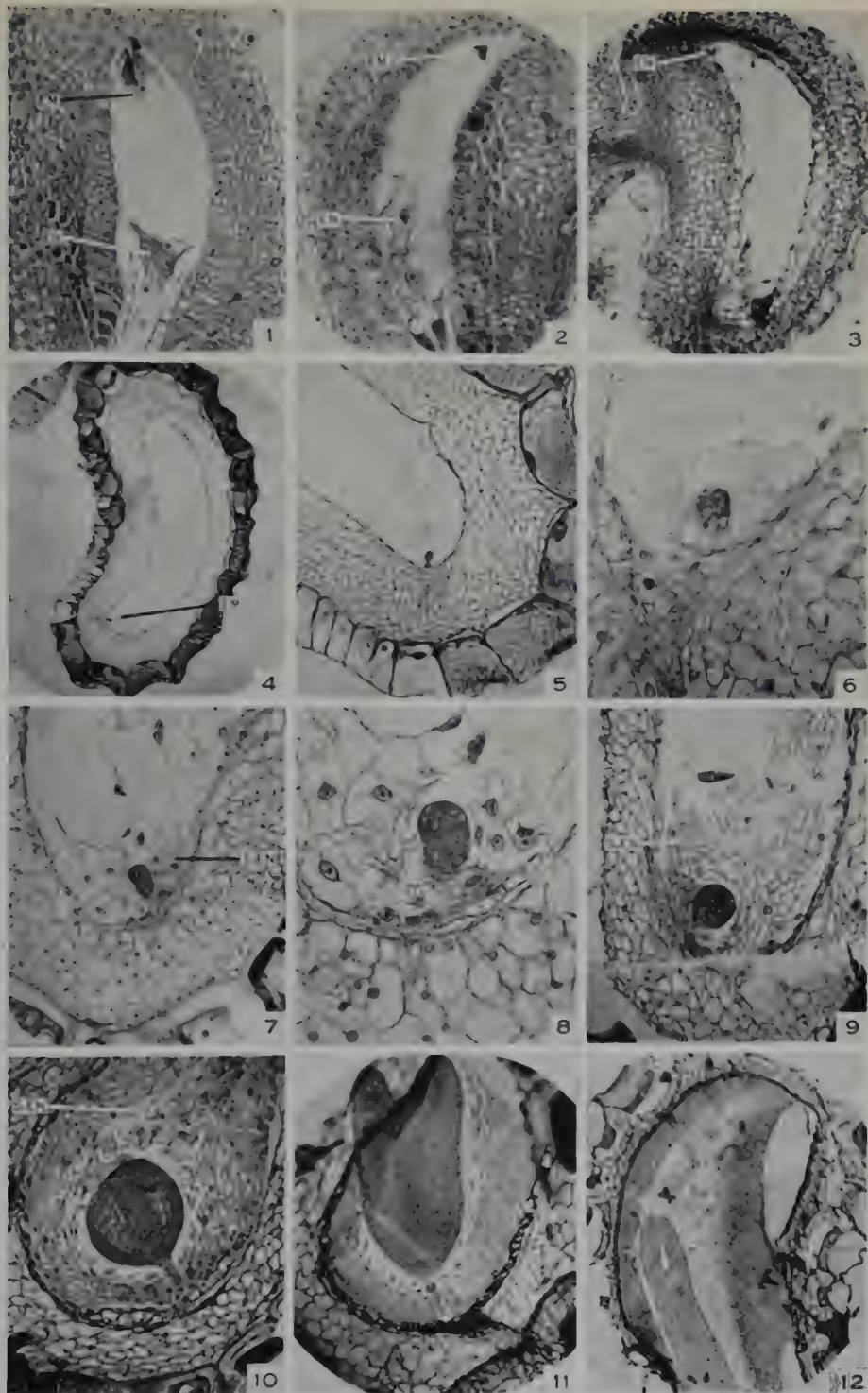
- Fig. 3.—L.S. ovule with single celled embryo at "EM" and cellular primary endosperm tissue. $\times 83$.
- Fig. 4.—L.S. ovule which has almost reached mature seed size. Few celled embryo at "EM." Endosperm tissue of large very delicate walled cells not visible in photograph. $\times 31$.
- Fig. 5.—More detailed view of micropylar end of ovule shown in Figure 4. $\times 83$.
- Fig. 6.—Detailed view of embryo at comparable stage to that shown in preceding photograph. Several of the thin-walled and large cells of the primary endosperm are visible. $\times 211$.
- Fig. 7.—Micropylar end of ovule in which endosperm nuclei in the vicinity of the embryo have divided to form a more dense compact tissue (S.EN). $\times 103$.
- Fig. 8.—Detailed view of embryo at about 14-15-celled stage with meristematic endosperm cells surrounding. $\times 251$.
- Fig. 9.—Showing secondary endosperm tissue (S.EN) filling up the embryo-sac and embryo at further stage of development. $\times 108$.
- Figs. 10-12.—Further stages in the development of the embryo. When the embryo has reached the stage shown in Figure 10 the secondary endosperm tissue has almost completely filled the sac and digestion of this tissue by the developing endosperm is definite. Figures 10 and 11 $\times 108$; Figure 12 $\times 34$.

PLATE 3

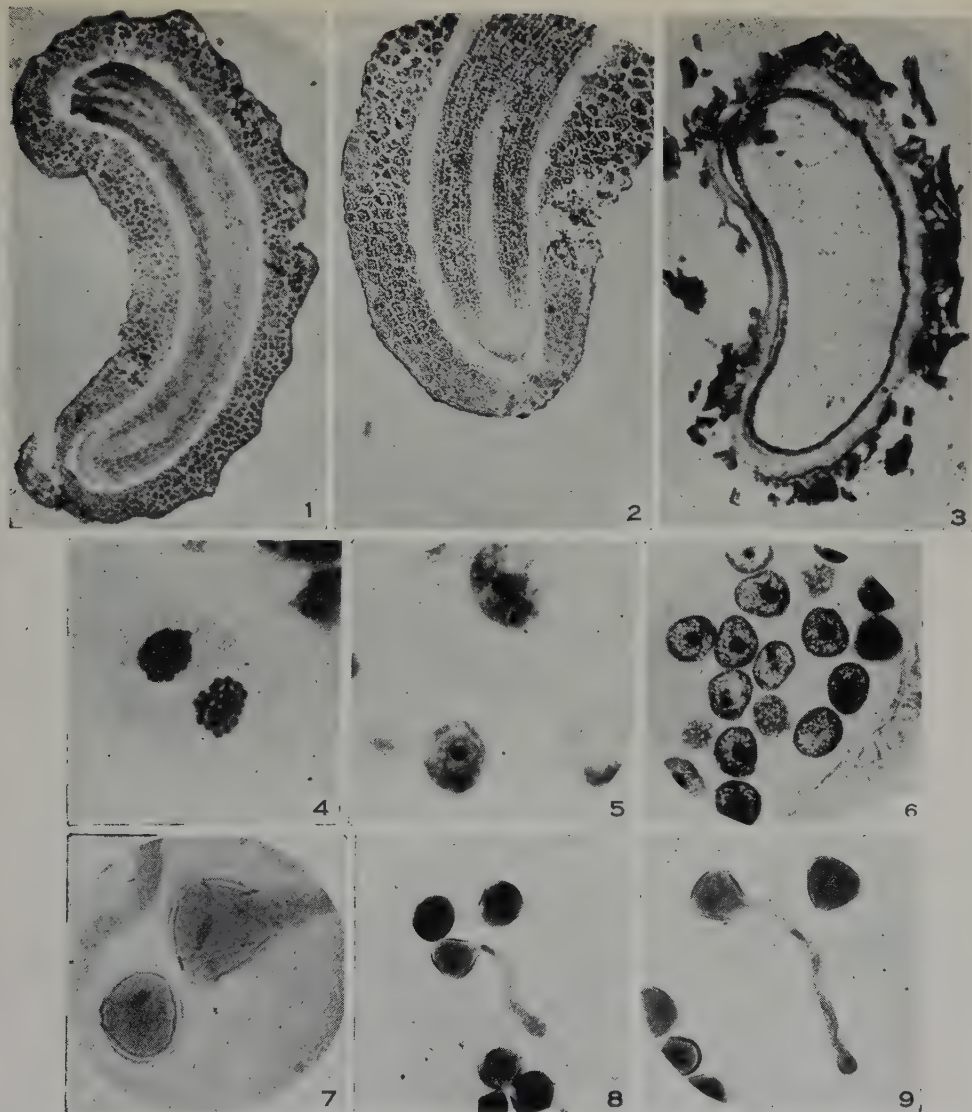
- Fig. 1.—L.S. of mature seed from which the testa has been removed showing suspensor of embryo and radicle end of the hypocotyledonary axis in detail. The cells of the dense endosperm tissue are filled with oil drops. $\times 33$.
- Fig. 2.—L.S. of mature seed from which testa has been removed. No epicotyl has been formed. $\times 33$.
- Fig. 3.—L.S. of an "empty" seed. No embryo nor secondary endosperm has been developed. The thin-walled cells of the primary endosperm contain practically no stored metabolites. $\times 48$.
- Fig. 4.—The metaphase plate of the second division of a pollen mother cell in which 30 chromosomes may be counted. $\times 1213$.
- Fig. 5.—Pollen grains in which the nucleus has divided to form a generative and vegetative nucleus. $\times 507$.
- Fig. 6.—Pollen grains at a later stage of development when they are filled with starch grains and the vegetative nucleus is degenerating. $\times 253$.
- Fig. 7.—Mature pollen grains showing absence of starch, the three germ pores and single nucleus and tube. $\times 507$.
- Figs. 8 and 9.—Germinating pollen grains showing behaviour of the single nucleus. $\times 253$.



BARNARD.— MICROSPOROGENESIS, MACROSPOROGENESIS, AND DEVELOPMENT OF THE MACROGAMETOPHYTE AND SEED OF *DUBOISIA LEICHARDTII* (F.V.M.) AND *D. MYOPOROIDES* (R.BR.)



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THE SIGNIFICANCE OF THE NECROTIC PHLOEM REACTION IN THE POTATO TO THE LEAF-ROLL VIRUS

By E. M. HUTTON*

(Plates 1-2)

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Summary

A modified technique which has facilitated the detection of phloem necrosis in leaf-roll-infected potato plants is described. Placed on a quantitative basis, the necrotic reaction has proved valuable in the estimation of leaf-roll severity in varieties and hybrids. Quantitatively, phloem necrosis has been shown to be a varietal character which can be influenced by nutritional and environmental conditions. Among varieties and hybrids, a range of necrotic reactions in the phloem from mild to very severe was found, and these reactions were correlated with external symptoms. Severe reactors show a progressive decrease in the amount of phloem necrosis from the stem base to the tip. It is probable that a severe physiological reaction to leaf roll has a well-defined genetic basis, so that the development of hybrids hypersensitive to this virus is possible. This provides a new approach to the problem of leaf-roll resistance in the potato.

I. INTRODUCTION

Depending on variety and growth conditions, the leaf-roll virus can reduce the yield of potato plants by 30 to 90 per cent. (Loughnane 1941; Le Clerg *et al.* 1944; Bald, Norris, and Helson 1946). Varietal reactions to this virus differ considerably and vary from relatively tolerant, as with Up to Date, through intermediate reactions to severe, as with President. Loughnane (1941), Clinch, Loughnane, and McKay (1944), Black and Cockerham (1943), Whitehead, McIntosh, and Findlay (1945), and Oortwijn Botjes (1947) have grouped common potato varieties according to their reactions to the leaf-roll virus, the extent of yield decrease being closely correlated with severity of reaction. Masking of leaf-roll symptoms can occur in varieties with tolerant and intermediate reactions when environmental conditions are conducive to vigorous haulm development, as on rich soils in a mild moist climate. These conditions rarely cause masking of symptoms in varieties which react severely to the leaf-roll virus. In districts where hot dry periods occur during the season, growth of potato plants is reduced and leaf rolling due to physiological stress is common, so that in these environments diagnosis of virus leaf roll can be difficult. The factors which affect the expression of leaf-roll symptoms in potato varieties have recently been studied by Felton (1948), who found that certain combinations of temperature, moisture, and soil fertility may delay the appearance or mask symptoms due to the leaf-roll virus.

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Of the four major potato viruses, leaf roll is now the most important in Australia. The existence of material with hereditary factors for resistance to viruses A, X, and Y (Cockerham 1943*b*; Cockerham and M'Ghee 1947; Hutton 1948) makes it possible to eliminate these viruses by genetic means. As yet no material with a well-defined hereditary resistance to the leaf-roll virus has been found. Müller (1939) produced leaf-roll-tolerant potato seedlings but was unable to develop resistant types. Several genes acting cumulatively for resistance to the leaf-roll virus were found in certain cultivated varieties by Cockerham (1943*a*). These factors were concentrated by inbreeding but were lost in outbreeding. In the United States, Stevenson, Folsom, and Dykstra (1943), Folsom and Stevenson (1946), and Locke (1948) failed to find leaf-roll immunity but showed that under field conditions some varieties and their progenies become less readily infected than others. Locke (1948) considered that the basis for field resistance was to be sought among the factors affecting the complex relationships between virus, host, vector, and environment. McKay and Clinch (1944) and Bald, Norris, and Helson (1946) demonstrated in the varieties Skerry Champion and Bismark respectively, a severe reaction to leaf roll resulting in high field resistance to this virus. The resistance problem is complicated by the nature of the leaf-roll virus, which differs from the other three major potato viruses in being non-sap-transmissible, serologically inactive (Chester 1937), persistent in aphid vectors (Smith 1931), and apparently confined to the phloem region (Bennett 1940).

The present paper records the results of a study in the greenhouse and field of the leaf-roll reactions of potato varieties and their seedlings. It is only by an understanding of these reactions that a basis for development of leaf-roll-resistant seedlings can be formulated.

II. MATERIALS AND METHODS

(a) *Potato Varieties Used*

A collection of old and modern varieties of potatoes from Australian and overseas sources was maintained free of all viruses except X, although it was possible to maintain some of the varieties in a virus-free condition. The varieties, and hybrids derived from a few of them, were used to study plant reactions to the leaf-roll virus in the greenhouse and field.

(b) *Inoculation Technique*

Sprout inoculation with the aphid species *Myzus persicae* proved to be the most satisfactory transmission method for studying current season reactions to the leaf-roll virus in potato material, as the early infection allowed full development of symptoms during growth. The procedure in sprout inoculation was to allow individuals of *Myzus persicae* a seven-day feeding period on leaf-roll-infected potato plants after which these aphids were transferred to sprouted setts, 10-12 aphids being placed on each sprout. After a seven-day feeding period on the sprouts, the aphids were killed with nicotine sulphate fumigation and the

potato setts planted. All sprout inoculations described were made from the same leaf-roll source, which was a Katahdin line containing this virus, but free from other viruses. This line was propagated from year to year under insect-free greenhouse conditions and always had well-defined leaf-roll symptoms.

(c) *Estimation of the Effect of Leaf Roll on the Above-ground Growth of Varieties in the Field*

The effect of leaf roll on the above-ground growth of varieties in the field was estimated by comparing the size of plants (Bald 1943) produced from equal numbers of sprout inoculated and healthy setts of each variety planted separately in alternate rows. A unit plot contained 12 to 18 plants, every fifth plot being planted with inoculated or healthy setts of the variety Factor, depending on whether the row was planted with infected or healthy setts. During the season, owing to natural aphid transmission, leaf-roll infection occurred in varying proportions of the plants from healthy setts. It was usually after flowering that natural infections affected plant growth, so size ratings were made at three 14-day intervals from emergence until flowering. When rating a group of plants, a visual size estimate of a large plant was made and the total leaf area with its true size rating was calculated by measuring each leaf with photographic leaf standards. This procedure was then repeated with five other plants ranging in size down to the smallest. If the estimated and true size ratings were in close agreement each plant in the block was given a size rating, six plants being checked at the finish from the standards to ensure that the ratings were made accurately.

(d) *Staining Technique for Detection and Estimation of the Severity of Leaf-Roll Infection*

Owing to varying climatic factors it was difficult to determine in the field whether a plant was infected with leaf roll or not, and masking of symptoms frequently occurred in the greenhouse, particularly in the more tolerant reactors. To test for the presence of leaf roll in all doubtful plants from both field and greenhouse experiments would involve considerable time and greenhouse space. The lack of a quick and accurate method of leaf-roll detection has retarded leaf-roll research for some time. It was found that the technique developed by Sheffield (1943) and improved by Wilson (1948) for the diagnosis of leaf roll from the occurrence of necrotic areas in the external or internal primary phloem of basal stem sections was a valuable aid in leaf-roll experiments. The main disadvantages of this improved technique were the irritation to the operator and the corrosion to the microscope resulting from hydrochloric acid fumes. It was found (Hutton 1948) that a combination of 40 per cent. sulphuric acid (v/v) and 2 per cent. phloroglucinol (w/v) in 50 per cent. alcohol (v/v) gave satisfactory results without objectionable fumes. A comparison between the hydrochloric acid and sulphuric acid methods with a series of sections from leaf-roll-infected plants showed that the latter, although it did not bleach the chlorophyll of fresh sections like hydrochloric acid, gave a brighter and more distinct staining as well as more consistent results. Leaf roll could be detected in over three-

quarters of the plants in which diagnosis was doubtful. The extent to which the external primary phloem was stained in infected plants varied from variety to variety and from sample to sample within a variety. To make comparisons between samples a method was developed for estimating the percentage of the external primary phloem region affected in hand-cut transverse stem sections viewed at a magnification of 110 diameters through the microscope. In well-developed stems, the external primary phloem could be distinguished as a continuous band of tissue bounded by scattered pericyclic fibres on the outside and by a dense layer of secondary phloem on the inside. In young or poorly-developed stems it was clearly defined only in the vascular bundles. Consequently the estimations were based on the proportion of stained tissue appearing in the external primary phloem region of the original vascular bundles. Estimates were made on a percentage basis to the nearest 5 per cent., the method being checked by means of a graticule placed in the eye-piece of the microscope. A 12-inch circle ruled with indian ink into squares by lines 0.1 in. apart was photographed and then reduced to a 1-cm. circle as the positive, the negative being developed as the graticule. The percentages of the external primary phloem region stained in several lots of 24 stem sections from infected plants were estimated and then checked with the graticule. It was apparent that the error of a subjective estimate had a maximum at 50 per cent. necrosis, the standard deviation at this point being of the order of 5 per cent., so that 95 per cent. of such estimates lay between 40 and 60 per cent. The standard error at other levels of necrosis is approximately $1/10\sqrt{p(100-p)}$ where p is the percentage of necrosis. The accuracy of the estimation method for phloem necrosis can be checked from time to time by means of the graticule.

In all sections examined, the extent to which the internal primary phloem was affected was noted but no attempt was made to estimate the percentage of necrosis occurring in this region. Instead, necrosis of the internal primary phloem was described in one of the four categories, absent, light, medium, or heavy.

III. PHLOEM NECROSIS IN LEAF-ROLL-INFECTED POTATO VARIETIES AND HYBRIDS

The nature and occurrence of phloem necrosis in leaf-roll-affected potato plants have been described by Artschwager (1923), Quanjer (1931), Bawden (1932), and Sheffield (1943). The reaction is usually well defined and quite distinct from top necrosis (Bawden 1932), virus Y (Hutton 1948) and potato rosette (Hutton and Oldaker 1949).

Sheffield's studies (1943) indicated the possibility of varietal differences in the amount of phloem necrosis. She also found that necrotic tissue could occur in either the external or the internal primary phloem or in both, but was at its maximum in the basal node at ground level and could extend vertically through the plant, the amount of necrosis being correlated with the severity of external symptoms.

The results of the above investigations show conclusively that primary phloem necrosis has high diagnostic significance. Large-scale work at Canberra

has confirmed this in general, but it has been found that leaf roll can occur without phloem necrosis being evident.

(a) *Phloem Necrosis as a Varietal Character*

In the 1946-47 season two series of potato varieties were planted at the Dickson Experiment Station, Canberra. The first series contained 10 setts of each of 54 varieties which were sprout inoculated just prior to planting. The second series comprised 12 setts of each of 20 varieties, different from those in the first series, which had been sprout inoculated before planting the previous season, and so had been infected with leaf roll for twelve months. Each sett in the second series represented one plant grown previously. In both series every fifth plot was a check plot of the variety Factor.

A week after flowering in the 1946-47 season, pieces of stem consisting of the three basal nodes were cut from each plant and stored in a 50 per cent. (v/v) solution of alcohol and water. Transverse stem sections from the basal node were later examined by the phloroglucinol-sulphuric acid technique and the percentage of necrotic external primary phloem was estimated. With the 54 varieties of the first series all but Arran Signet, Field Marshal, Pink Eye, Red Maclure, and Rural New Yorker had varying numbers of plants without necrosis. Over all the varieties, with the exceptions mentioned, 43.3 per cent. of the plants had no phloem necrosis. When these non-necrotic plants were disregarded, the plants in 55 per cent. of the varieties had amounts of necrotic tissue in the external primary phloem within 10 per cent. of the mean. The rest of the varieties showed considerable plant-to-plant variation in the extent of phloem necrosis. These points are illustrated by the four varieties shown in Table 1. The 12 Factor check plots had much the same plant-to-plant variation as the Factor in Table 1.

TABLE 1

ESTIMATED PERCENTAGES OF NECROTIC TISSUE IN THE EXTERNAL PRIMARY PHLOEM OF EACH OF 10 LEAF-ROLL-INFECTED PLANTS OF 4 POTATO VARIETIES

Variety	Percentages of Phloem Necrosis in Each 10 Plants										Mean
Rural New Yorker	20	20	30	20	20	20	20	20	20	20	21
Arran Signet	30	70	40	40	40	90	70	40	30	90	54
Factor	20	20	10	0	0	20	10	10	20	20	13
Great Scot	30	0	80	90	50	70	10	80	50	0	46

In contrast to these results of the first series, plant variability with respect to phloem necrosis within the varieties of the second series, infected with leaf roll a season before planting, was greatly reduced. This was also reflected in the plants of the Factor check plots. Among the second series, except in varieties with a mild phloem reaction, there were very few plants with no necrosis or with amounts of necrosis differing by more than 10 per cent. from the mean. These results indicate that the leaf-roll virus is one which takes some time to reach equilibrium with its host. This may be due to the restriction of its vital activities to the phloem region. Statistical analysis of the data from the second series

showed significant varietal differences in the amount of necrotic tissue present in the external primary phloem. Table 2 lists the varieties in order of the mean percentage of the external primary phloem region affected as a result of leaf-roll infection. It is apparent from the results shown in Table 2 that under the condi-

TABLE 2
MEAN PERCENTAGES (BASED ON 12 PLANTS) OF THE EXTERNAL PRIMARY PHLOEM REGION AFFECTED IN 20 POTATO VARIETIES GROWN IN THE 1947-48 SEASON AT THE DICKSON EXPERIMENT STATION

Variety	Necrosis of External Primary Phloem (%)	Variety	Necrosis of External Primary Phloem (%)
President	72	Sequoia	29
Pawnee	68	Sussex Red	27
Pioneer Rural	50	Delaware	27
Snowflake	50	Inverness Favourite	25
Late Carman	45	Red Dakota	24
Mohawk	38	Robin Adair	20
Katahdin	38	Brownell	20
Brown's River	31	Potomac	20
Kasota	26	Factor	13
Sebago	30	North Carolina	13

Min. diff. for significance at 5% level = 6.2% phloem necrosis.

tions of this experiment sensitivity of the primary phloem, as measured by the extent of necrosis, is a varietal factor. These results provide concrete evidence for Sheffield's (1943) suggestion that varietal differences in the amount of phloem necrosis may occur. Plate 1, Figure 1, is a photomicrograph of a section from the base of the stem of a leaf-roll-infected Katahdin plant showing the stained areas in the external primary phloem. It is interesting to note that varieties like President, Snowflake, and Late Carman, which rarely exhibit masking of leaf-roll symptoms in field crops, had half or more of the external primary phloem area necrosed in infected plants. On the other hand, Brownell and Factor, which frequently show masking of leaf-roll symptoms, had one-fifth or less of the external primary phloem affected. On this basis, masking of symptoms could be expected to occur at times in varieties like Sebago, Sequoia, and Delaware, and field experience has borne this out.

The general conclusion to be drawn from the work described is that, in order to obtain reliable data on varietal differences in the incidence of phloem necrosis, one must use potato material which has been infected with leaf roll for a growing season before planting. The longer infection period appears to allow the virus time to attain equilibrium with its host.

(b) The Influence of Environmental Conditions and Time of Sampling on the Incidence of Phloem Necrosis

A further experiment has also shown the necessity of taking environmental conditions into account when considering the incidence and intensity of phloem

necrosis. A small greenhouse experiment with 5 setts from the same tubers as used for 12 of the varieties shown in Table 2 and grown during the winter of 1947 gave the results tabulated in Table 3.

TABLE 3

MEAN PERCENTAGES (FROM 5 PLANTS) OF THE EXTERNAL PRIMARY PHLOEM REGION AFFECTED IN 12 OF THE VARIETIES OF TABLE 2 GROWN IN THE GREENHOUSE

Variety	Necrosis of Primary Phloem (%)	Variety	Necrosis of Primary Phloem (%)	Variety	Necrosis of Primary Phloem (%)
Katahdin	55	Snowflake	23	Delaware	8
Late Carman	52	Mohawk	20	Brown's River	7
Pioneer Rural	33	Brownell	18	Factor	7
Kasota	27	Sebago	10	North Carolina	4

The results in Table 3 compared with those in Table 2 show that the percentage of necrosis in the external primary phloem of a potato variety is influenced by environmental conditions, although it is possible, as with Kasota and Brownell, for it to remain constant. In eight of the varieties in Table 3 the amount of necrosis has decreased when compared with Table 2, the reduction varying from as much as 20 per cent. in Sebago to as little as 6 per cent. in Factor. With Katahdin and Late Carman there was an increase in the percentage of necrosis. These variations between field and greenhouse material may be due to the more irregular phloem development and smaller stem diameter of the latter. The same kind of variation could be expected from samples of a variety grown in different districts. The fact that phloem necrosis is influenced by environmental conditions does not, however, detract from the value of the observation that variety is one of the major factors governing this reaction.

(c) *The Effect of Nutritional Conditions on the Extent of Phloem Necrosis in Six Leaf-roll-infected Varieties*

From the results in Tables 2 and 3 the inference was drawn that there was a tendency for the better soil and moisture relationships in the greenhouse to result in a decrease in the amount of phloem necrosis in leaf-roll-infected varieties. To test this, lines of the varieties Bismark, Brownell, Delaware, Factor, President, and Snowflake which had been infected with leaf roll for four seasons were selected. These lines were infected with virus X but were free from viruses A and Y. Ten tubers of each of these lines were planted in the greenhouse during the winter. One half of each tuber was planted in a pot of washed river sand and the other half in a pot of rich loam. Ten weeks after emergence, when tuberization was advanced, the height of each plant was measured and the bases harvested and examined for the occurrence of phloem necrosis. Plate 1, Figure 2, shows the comparison between the plants from two halves of one of the Bismark tubers grown in sand and soil respectively.

The results are given in Table 4. As would be expected, growth measured as mean height in inches is significantly less in sand than in loam. Leaf-roll symptoms were obvious in the sand series but in the loam series they were indeterminate or masked in the varieties Bismark, Brownell, Delaware, and Factor. The only two varieties with well-defined leaf-roll symptoms in loam were President and Snowflake. These results are interesting as they show that nutritional conditions can affect the expression of leaf-roll symptoms, and that Bismark is able to react tolerantly to leaf roll like Factor in loam, and sensitively like President in sand.

TABLE 4
THE EFFECT OF LOAM AND SAND ON THE HEIGHTS AND AMOUNTS OF PHLOEM NECROSIS OF PLANTS GROWN FROM THE SAME TUBERS OF 6 LEAF-ROLL-INFECTED VARIETIES

Variety	Mean Height (in.)		Difference between Mean Heights	Mean Percentage Necrosis in External Primary Phloem		Difference between Mean Percentage Necrosis
	Loam	Sand		Loam	Sand	
Bismark	12.8	3.8	9.0	8.5	12.5	4.0
Brownell	15.3	7.5	7.8	15.0	27.5	12.5
Delaware	19.4	6.8	12.6	5.5	25.0	19.5
President	8.8	3.7	5.1	20.0	23.3	3.3
Snowflake	6.5	3.2	3.3	15.0	24.2	9.2
Factor	12.5	5.8	6.7	9.3	20.0	10.7
Min. diff. for significance at 5% level	2.2	1.1	2.6	5.2	5.8	7.1

When the percentage necrosis in the external primary phloem area is considered, it can be seen from Table 4 that in loam most of the varieties are significantly different from each other, whereas in sand, necrosis is at a higher level, and with the exception of Bismark, differences between varieties are barely significant. The differences between sand and loam are small and non-significant in Bismark and President, but are significant in the other varieties, the greatest difference occurring in Delaware. These results make it clear that nutritional conditions are a factor along with variety in determining development of necrosis in the external primary phloem of leaf-roll-infected potato plants.

(d) *The Distribution of Phloem Necrosis in Stems of Leaf-roll-infected Plants of Potato Hybrids and the Variety President*

To investigate the extent to which phloem necrosis spreads in the stems of leaf-roll-infected potato plants, 2 tubers from each of 12 President plants and 3 tubers from each of 22 hybrids, field grown at the Dickson Experiment Station from sprout-inoculated setts, were planted in the greenhouse during the winter. Six weeks from emergence, stems were harvested from each plant and the percentage necrosis in the external primary phloem estimated, sections being taken from the basal node, mid-stem, and tip.

The hybrids could be divided into two groups according to the amount of necrosis occurring in the external primary phloem. In the first group, containing 10 hybrids, phloem necrosis was either absent or present to the extent of 1 to 5 per cent. in the plants, with the occasional occurrence mainly in basal sections of a light necrosis of the internal primary phloem. Six of these hybrids were symptomless or had indeterminate leaf-roll symptoms while the other four had well-defined symptoms. The six hybrids with masked symptoms when grown under summer conditions had obvious although not severe leaf-roll symptoms. It has been observed repeatedly that masking of leaf-roll symptoms occurs more often in the greenhouse under winter than under summer conditions, probably because of the shorter photoperiod and lower temperatures of winter.

The plants from the second group of 12 hybrids and 10 of the President lines had well-developed phloem necrosis. The other 2 President lines represented by 4 plants averaged only 1 per cent. necrosis of the external primary phloem and had a light necrosis of the internal primary phloem. All the President plants had definite leaf-roll symptoms. Table 5 summarizes the results from the second group of hybrids and the 20 President plants with well-developed phloem necrosis.

TABLE 5
DISTRIBUTION OF PHLOEM NECROSIS IN THE STEMS OF LEAF-ROLL-INFECTED HYBRIDS AND PRESIDENT

	Mean Phloem Necrosis of the External Primary Phloem (%)			Plants with Necrosis of the Internal Primary Phloem (%)		
	Base	Mid-Stem	Tip	Base	Mid-Stem	Tip
12 hybrids (36 plants)	30	14	1	100	77	21
20 President plants	41	4	0	100	67	1

Table 5 shows clearly that in plants with well-developed phloem necrosis there is a gradation in the amount of necrosis from the base to the tip, the occurrence of necrosis of the internal primary phloem following much the same pattern. These results are in agreement with Sheffield's (1943) observation that phloem necrosis is usually at a maximum in the basal nodes of infected plants. There were a few cases among the hybrids of plants with a higher percentage of necrosis in mid-stem sections than in basal sections.

Of the group of 12 hybrids shown in Table 5, half exhibited masking of leaf-roll symptoms, none of these plants having more than 30 per cent. of the external primary phloem affected. Among the rest of the hybrids in group 2, all of which had definite leaf-roll symptoms, four had greater than 50 per cent. necrosis and two had 15 per cent. and 30 per cent. necrosis respectively in the external primary phloem.

It is apparent that although there is a correlation between the severity of leaf-roll symptoms and the extent of phloem necrosis, this correlation does not always hold, as it is possible to have relatively severe external symptoms together with a low level of phloem necrosis.

IV. THE EFFECT OF THE LEAF-ROLL VIRUS ON THE FIELD GROWTH OF POTATO VARIETIES

All measurements of the effect of leaf roll on the field growth of potato varieties were made at the Dickson Experiment Station, Canberra. Here potato plots received irrigation as required so that growth was satisfactory and comparable with the better crops grown in the Southern Highlands of New South Wales. In the 1947-48 season two series of plots were planted, one from a group of varieties which had been sprout inoculated with leaf roll before planting the previous season, and one from a group of varieties which were sprout inoculated before planting. Twelve varieties were common to both series. At the same time plants of the same varieties not infected with leaf roll were planted in rows alternating with the infected ones. Plot arrangement and the method used for rating the size of plants were as described in Section II (c).

Table 6 summarizes the grouping of the varieties from the first series in which the growth of plants from varieties infected with leaf roll for a season before planting was compared with the growth from varieties healthy at planting. The grouping was based on the third rating done when the plants were in flower. It can be seen from Table 6 that the ratio between the sizes of plants from infected and healthy setts tended to be medium to high in most of the varieties tested. Only 5 of the 26 varieties were classed as being markedly depressed in growth owing to leaf-roll infection. Masking of leaf-roll symptoms is most likely to occur in the group of varieties showing least growth depression. In the second series the 31 varieties which were sprout inoculated just prior to planting showed a less decided growth depression generally than the first series. The ratios of the sizes of plants from infected and healthy setts were all above 60 per cent. Six varieties had ratios between 60 and 70 per cent., 12 were between 75 and 85 per cent., and the rest were over 90 per cent. The Factor checks were all in this last group although the ratios of the 12 varieties common to both groups did not always have a consistent relation to their ratios in the first series. It is apparent that growth depression due to leaf roll is generally more marked in the second than in the first season after sprout infection. As stated previously, phloem necrosis tended to follow a similar pattern. Where estimates of the extent of phloem necrosis in stems were made in the first series the tendency was for those varieties with the greatest growth depression to have the highest percentage of necrotic phloem tissue.

In order to investigate the effect of relatively long-standing leaf-roll infections on the growth of varieties, size ratings were made on field-grown plants of the varieties Bismark, Brownell, Delaware, Factor, Katahdin, President, and Snowflake grown from setts infected with leaf roll for one season and four seasons respectively. These ratings were compared with those from plants of the same varieties grown from healthy setts. The results are given in Table 7, and it can be seen that with Brownell, Factor, President, and Snowflake, setts infected with leaf roll for four seasons before planting gave plants with similar growth to setts infected for one season. With Bismark and Delaware and to a lesser extent with

Katahdin, there was a significant decrease in growth from setts infected for four seasons compared with growth from setts infected for one season. It appears that under the conditions of these experiments, depression of growth due to leaf

TABLE 6

GROWTH DEPRESSION DUE TO LEAF ROLL MEASURED AT FLOWERING BY THE RATIO BETWEEN THE SIZES OF THE PLANTS FROM INFECTED AND HEALTHY SETTS RESPECTIVELY. VARIETIES OF SERIES I GROUPED INTO THREE RATIO CLASSES: LOW, MEDIUM, AND HIGH

30-40% (Low)	45-55% (Medium)	60-70% (High)
Early Carman	Brown's River	Bismark
Late Carman	Golden Wonder	Brownell
Mohawk	Inverness Favourite	Delaware
President	Kasota	Epicure
Robin Adair	Katahdin	Factor (Up to Date)*
	King Edward	Houma
	Red Dakota	Pawnee
	Smooth Rural	Pioneer Rural
	Snowflake	Potomac
		Sebago
		Sequoia
		Sussex Red

* The Factor checks were consistently placed in this class.

roll reaches an equilibrium in some varieties after one year's infection, whereas in other varieties loss of vigour tends to progress according to the number of years they have been infected. As shown later, Bismark tends to have a variable

TABLE 7

SIZE RATINGS OF PLANTS OF 7 POTATO VARIETIES FIELD GROWN FROM HEALTHY SETTS AND SETTS INFECTED WITH LEAF ROLL FOR ONE YEAR AND FOUR YEARS RESPECTIVELY

Variety	Setts Healthy*			Setts infected 1 Season†			Setts infected 4 Seasons†		
	1st Rating	2nd Rating	3rd Rating	1st Rating	2nd Rating	3rd Rating	1st Rating	2nd Rating	3rd Rating
Bismark	3.6	11.3	14.6	2.7	5.6	8.8	1.9	3.8	4.2
Brownell	2.5	7.8	12.1	1.8	5.3	7.3	1.7	4.6	6.9
Delaware	3.8	11.9	14.3	2.7	7.2	9.4	1.5	4.1	5.8
Factor	4.3	9.0	13.8	4.1	6.3	8.7	3.0	5.8	9.0
Katahdin	4.2	10.3	13.3	2.4	5.9	7.2	1.7	3.8	5.0
President	3.3	9.3	14.1	1.7	4.6	5.6	1.9	4.4	5.9
Snowflake	2.5	7.5	14.1	1.7	4.9	7.2	3.2	5.4	6.9
Min. diff. for significance at 5% level	0.71	0.91	0.87	0.64	0.84	0.79	0.58	0.75	0.71

* 12 setts planted. † 18 setts planted.

leaf-roll reaction, so that if mildly reacting instead of severely reacting lines had been used the results of this experiment in relation to this variety might have been different.

V. THE LEAF-ROLL REACTIONS OF SOME VARIETIES STATED TO BE FIELD RESISTANT TO THIS DISEASE

Immunity to leaf roll in a potato variety has not yet been discovered. None of the varieties tested during the course of the work reported in this paper has withstood infection with leaf roll by the sprout inoculation technique described in Section II(b). This indicates the lack of a clearly defined physiological resistance to leaf roll in existing potato varieties. It is only under the variable conditions of field infection that differences in the leaf-roll susceptibilities of varieties become apparent (Loughnane 1941; Stevenson, Folsom, and Dykstra 1943; Locke 1948) and even in the field the indications are that heavy aphid infestations reduce differences in leaf-roll resistance between varieties (Whitehead and Currie 1931; Locke 1948).

In Ireland, McKay and Clinch (1944) have shown the variety Skerry Champion to be highly resistant in the field to leaf roll, and in Australia, Bald, Norris, and Helson (1946) have described a similar type of resistance in the variety Bismark. Among common varieties, Arran Banner and Majestic (Loughnane 1941; Bawden and Kassanis 1946), Katahdin (Locke 1948), and Epicure (Salaman 1926) have been shown to possess a relatively high field resistance. Cockerham has listed for Whitehead, McIntosh, and Findlay (1945) the leaf-roll susceptibilities and reactions of 64 potato varieties. In this list Arran Crest, Champion, and International Kidney are classified as being "fairly resistant" and when infected as having "fairly severe" or severe symptoms. Among the susceptible or very susceptible varieties, Arran Signet and President are classified as having very severe symptoms when infected, while Great Scot and Up to Date are listed as having "fairly tolerant" reactions. It is apparent from the work quoted that potato varieties vary greatly not only in their susceptibilities to leaf roll but also in their reactions to it. The ideal variety in this respect would be one with a high resistance to initial infection together with a severe reaction once infection has been established. A severe reaction after infection is necessary to preclude the possibility of masking of leaf-roll symptoms.

As a preliminary to the initiation of a programme for the development of resistant hybrids, the leaf-roll reactions of some varieties which have been reported as resistant were investigated in order to assess their suitability as parents. Twenty-five tubers of Bismark, Epicure, and Skerry Champion and 12 each of Arran Crest, Arran Signet, Factor, and President, the latter 3 varieties being included as checks, were sprout inoculated with leaf-roll-infected aphids and then planted at the Dickson Experiment Station in the 1946-47 season. Before inoculation, the tubers were virus-free except for virus X in Skerry Champion and Arran Signet. Twenty-five virus-free and non-inoculated Bismark tubers were also included in the trial. At flowering, the Epicure, Skerry Champion, and Factor plants were vigorous, with indefinite leaf-roll symptoms. In contrast, the Arran Crest, Arran Signet, and President plants had severe leaf-roll symptoms. The reaction of the sprout-inoculated Bismark was interesting, as 17 of the plants were large and vigorous and appeared healthy while the other 8 were small, with severe leaf-roll symptoms. When basal stem sections were examined

by the modified Sheffield technique, phloem necrosis varying in extent from 10 to 90 per cent. was found in all the Epicure, Arran Crest, Arran Signet, and President plants and also in the 8 Bismark plants with severe reactions. In comparison, no phloem necrosis was found in Skerry Champion while in Factor and the 17 vigorous Bismark plants it was either absent or present in amounts varying from 1 to 20 per cent. Of the 25 Bismark plants from uninoculated tubers, although all were large and vigorous, 8 had 10 to 20 per cent. phloem necrosis and 2 had traces, showing that a fair proportion of them had become field infected with leaf roll under the conditions of the experiment. The results from the sprout-inoculated series indicated a fairly close relationship between external symptoms and the internal reaction as measured by extent of phloem necrosis, although Epicure appeared to be a variety which could have a severe internal reaction together with relatively mild symptoms. Tuber samples were kept from all sprout-inoculated lines in the experiment and were later planted in the greenhouse.

The results in the greenhouse closely paralleled those previously obtained in the field. The plants of Arran Crest, Arran Signet, and President had both a severe external and a severe internal leaf-roll reaction, while Epicure had relatively mild symptoms but a uniformly high percentage of phloem necrosis, averaging 62 per cent. The Skerry Champion plants were fairly vigorous and could not be classified as having leaf roll. When basal stem sections were examined, all the Skerry Champion plants were found to have phloem necrosis which averaged 1 per cent. in extent. When the plants of this variety were allowed to regrow after cutting back, it was found that the regrowth had severe leaf-roll symptoms. Apparently the leaf-roll reaction in Skerry Champion is basically a mild one, but can be severe under lowered nutritional conditions. With Bismark the same variable leaf-roll reaction was evident among the tuber lines, so a second greenhouse planting was made two months later from the field samples followed by a further planting from them at the Dickson Experiment Station. Table 8 summarizes the results.

It can be seen from Table 8 that the variable leaf-roll response of Bismark tended to be retained as a clonal characteristic. Of the group with severe symptoms all maintained a high percentage of phloem necrosis, and only one line in the second greenhouse test and again in the second field trial was classed as having indefinite leaf-roll symptoms. Apparently this clonal line had unusual vigour which resulted in masking of symptoms in spite of a high percentage of phloem necrosis. Of the group with mild symptoms in Table 8, 5 out of the 17 lines could be classed after the first greenhouse test as having severe symptoms externally and internally. This was substantiated in the second greenhouse test and the second field planting. The remaining 12 lines of the mildly reacting group had definite although not severe symptoms at both or one of the greenhouse plantings, whereas in the second field planting only 2 had definite leaf-roll symptoms. Of these 12 lines, 6 had 5 to 10 per cent. phloem necrosis in the first greenhouse test, while the rest had none. The 6 former lines again showed a low

level of phloem necrosis in the second greenhouse planting while at the field planting 2 had a high percentage of phloem necrosis, 3 had 5 to 10 per cent., and 1 had a trace. Of the 6 which had no phloem necrosis in the first greenhouse

TABLE 8
EFFECT OF LEAF-ROLL INFECTION IN THE VARIETY BISMARK

Planting	Group with Mild Symptoms (17 plants)*			Group with Severe Symptoms (8 plants)†		
	No. without Leaf-Roll Symptoms	No. without Phloem Necrosis	Mean Phloem Necrosis (%)	No. without Leaf-Roll Symptoms	No. without Phloem Necrosis	Mean Phloem Necrosis (%)
Field from sprout- inoculated tubers	17	8	6	0	0	66
First greenhouse	4	6	12	0	0	36
Second greenhouse	5	4	—	1	0	—
Second field	10	3‡	22	1	0	42

* Mean yield per plant, 2041.8 g.

† Mean yield per plant, 290 g.

‡ Traces only of phloem necrosis.

test, 2 had 5 and 10 per cent. respectively in the second greenhouse test, while in the second field planting these 2 and 2 others had 2 to 5 per cent. phloem necrosis, the remaining 2 having a trace. When plants in the mildly reacting Bismark lines were examined for the presence of phloem necrosis, staining was sometimes indefinite in basal sections but clearly defined in mid-stem sections.

The apparent clonal reaction to leaf roll which occurred in the variety Bismark in these experiments is difficult to explain but has been confirmed under Tasmanian conditions by Wilson (1948)*. It may be owing to varying soil conditions some lines have developed an inherent physiological vigour which lasts for some time and causes masking and depression of symptoms for a few tuber generations. In addition, it is possible that Bismark is able in some way to separate out mild and severe strains from leaf roll. Whatever the explanation, it appears from these experiments that severely affected plants usually produce the same type of progeny while tolerant plants tend to produce tolerant progeny. It has been shown earlier in this paper that in Bismark lines severely affected with leaf roll a gradual reduction in vigour occurs from year to year in field plantings, while in the greenhouse, given the right nutritional conditions, such lines tend to give indefinite symptoms.

In a further greenhouse experiment during early summer, 30 leaf-roll-free tubers of Bismark and 24 of Skerry Champion were sprout inoculated with leaf-roll-infected aphids and planted. Twenty-one days after emergence all the Bismark and Skerry Champion plants had definite leaf-roll symptoms, but in another month, although symptoms in the latter variety were fairly definite, only

* Personal communication.

two Bismarks could be classed as having leaf roll, the rest having indefinite symptoms. Examination of the stem bases showed the presence of phloem necrosis in all the Skerry Champion plants, the average being 7 per cent. In the Bismark plants, 8 had no phloem necrosis, 12 had a low percentage, 5-15 per cent., and 10 had a high percentage, 40-60 per cent.

From these results it is apparent that varieties can have a stabilized or variable reaction to leaf roll. Varieties like President and Arran Signet with a stabilized and severe leaf-roll reaction are in direct contrast to a variety like Skerry Champion with a mild leaf-roll reaction. Bismark with a severe or mild leaf-roll reaction provides an example difficult to explain. It was considered that varieties with either a basically mild or variable leaf-roll reaction judged both externally and internally would not make good parents for the development of leaf-roll-resistant hybrids, as it was doubtful whether such reactions had a well-defined genetic basis. Even if such reactions were transferred to progeny by breeding, it seemed probable that they would not lead to resistance. On the other hand, a stabilized and severe physiological reaction to leaf roll as found in varieties like Arran Signet and President would be likely to have a well-defined genetic basis. Although this has not resulted in resistance in these two varieties, it has resulted in a measure of resistance in the variety Arran Crest. As the potato is heterozygous genetically, it is conceivable that seedlings more sensitive to leaf roll than the varieties quoted could be developed. Such seedlings with extreme leaf-roll sensitivity may in the field localize the leaf-roll virus owing to a rapid and severe necrotic reaction in the phloem following feeding by infective aphids. If the virus were not localized it would be possible for the reactions of any tuber progeny to be almost lethal. A lethal reaction to leaf roll has already been described by Oortwijn Botjes (1947).

VI. PROBLEMS ASSOCIATED WITH THE DEVELOPMENT OF LEAF-ROLL-RESISTANT POTATO HYBRIDS

Up to the present the work done overseas on the development of leaf-roll-resistant hybrids has not been particularly successful. Müller (1939) in Germany found it impossible to develop resistant hybrids and concentrated on the production of leaf-roll-tolerant progeny. Cockerham (1943*a*) was unable to establish a clearly-defined genetic basis for resistance and Locke (1948) in the United States considered that progress towards the development of leaf-roll-resistant varieties would continue to be slow and tedious until more was known about the nature of resistance. It is apparent from the work quoted and that presented in this paper that a new approach to leaf-roll resistance breeding is needed. As has been stated earlier, the possibility that leaf-roll sensitivity and resistance could be complementary factors needed investigation, as it provided a basis for work which might lead to more definite results. Over the last two years the Canberra work has aimed at evaluating the suitability of some varieties for the development of leaf-roll-sensitive hybrids and at finding the best means of measuring the relative sensitivities of any hybrids chosen. When a number of leaf-roll-sensitive

hybrids have been developed the next phase planned is to observe their reactions and susceptibilities to leaf roll under conditions of field infection for a period of years.

As some Bismark lines had been reported by Bald, Norris, and Helson (1946) as possessing both leaf-roll sensitivity and leaf-roll resistance, five crosses were made with this variety as one parent. At the same time Bismark was self-pollinated and a cross made between Snowflake and Katahdin for comparison. Snowflake is a variety which has a well-defined and fairly severe reaction to leaf roll both externally and in the phloem, and at the same time has a degree of resistance under field conditions. Katahdin has been described as being field resistant to leaf roll (Locke 1948), and although it tends to exhibit masking of symptoms under fertile growing conditions, it usually has a fairly severe phloem reaction when infected. A tuber from each of the seedlings raised from the crosses and inbred was later sprout inoculated with infective aphids and planted in the greenhouse. At the flowering stage the seedlings were classified into three groups — severe, medium, and indefinite — according to the severity of their leaf-roll symptoms. Those with severe symptoms were stunted, with small, yellow, frequently rolled leaves often showing necrotic lesions and the development of anthocyanin. The seedlings with symptoms of medium intensity were almost normal in size, most of the leaves, especially those of the basal half of the plants, being rolled and having various amounts of interveinal yellowing. Seedlings in the group with indefinite or masked symptoms grew vigorously, rolling and interveinal mottling of leaves being almost entirely confined to the base of the plants. Some of the seedlings of this last group had no discernible leaf-roll symptoms.

Table 9 summarizes the results, which indicate that the genes in Bismark conditioning sensitivity to the leaf-roll virus tend to have a low frequency, as the number of severe reactors in the progenies of the crosses involving this variety are of a low order and even its selfed progeny show no marked increase in the number of severe reactors. The severe reactions of a number of the inbred Bismark seedlings may have resulted from a decrease in vigour following inbreeding. It is interesting to note the absence of severe reactors in the cross between the leaf-roll-tolerant Factor and Bismark. By comparison with the Bismark crosses, the highest percentage of severe and the lowest percentage of medium reactors were found among the progeny of the Snowflake-Katahdin cross. The percentage of mild or indefinite leaf-roll reactors in Table 9 did not vary greatly from cross to cross.

The tuber progeny of most of the seedlings shown in Table 9 were later grown on again in the greenhouse. With a few exceptions, the seedlings could again be classified in the same categories as previously. Half the seedlings with indefinite symptoms in each cross were retained and side grafted with Katahdin scions containing leaf roll. The grafted plants were harvested and when the tubers from them were grown on again later in the greenhouse 75 per cent. of the resulting plants had mild or indefinite symptoms, the rest having symptoms of

medium intensity. This proved that sprout inoculation had infected them initially and that they were not resistant to leaf roll but were tolerant reactors to this disease.

TABLE 9

CLASSIFICATION OF THE LEAF-ROLL SYMPTOMS IN THE PROGENY OF BISMARK CROSSES IN COMPARISON WITH THOSE IN A SNOWFLAKE-KATAHDIN CROSS

Cross	No. of Seedlings	Severe (%)	Medium (%)	Mild or Indefinite (%)
Snowflake x Katahdin	468	28.8	40.0	31.2
Bismark inbred	104	14.4	53.0	32.6
Bismark x (B.R. x Kat.)*	92	9.8	70.0	20.2
Delaware x Bismark	76	13.2	67.2	19.6
Factor x Bismark	25	0.0	60.0	40.0
Bismark x (S.F. x Kat.)*	84	4.8	56.0	39.2

* Two seedlings, (Brown's River x Katahdin) and (Snowflake x Katahdin) respectively, with leaf-roll reactions of medium intensity.

Virus-free duplicate material of most of the seedlings shown in Table 9 to be severe leaf-roll reactors were sprout inoculated together with President, Arran Signet, and Factor checks and planted in the field at the Dickson Experiment Station. Five tubers of each seedling and 10 of each variety were planted and at flowering symptoms were noted and a basal stem sample from each plant was examined for the occurrence of phloem necrosis. The results from the Bismark crosses and inbreds were very variable, 60 per cent. of the lines having uniformly vigorous plants and the rest containing plants of variable vigour. Phloem necrosis showed a similar variation: no plant had more than 50 per cent. and in many it was absent. From these results it was difficult to classify any of the inbred or crossed Bismark seedlings definitely as severe or sensitive leaf-roll reactors. The Factor checks were all vigorous plants with amounts of phloem necrosis varying from traces up to 20 per cent. In contrast, all the President and Arran Signet plants were severely affected and had phloem necrosis which varied from 30 to 90 per cent. With only five exceptions, the seedlings from the Snowflake-Katahdin cross had uniform and definite reactions to leaf roll similar to that found in the Snowflake parent. Only 40 per cent. of these had a severe necrotic reaction in the phloem similar to that found in President and Arran Signet.

It was apparent from these results and those of Table 9 that Bismark and other varieties with a variable or mild reaction to leaf roll are unsuitable parents for the production of leaf-roll-sensitive seedlings. With more suitable parents like Snowflake and Katahdin the chances of obtaining seedlings with the combination of a severe external and internal reaction to leaf roll, as in President and Arran Signet, are greatly enhanced.

Following the results from the Snowflake-Katahdin cross and owing to the heterozygous nature of the potato it was considered unnecessary to introduce Arran Signet and President into crosses in order to develop seedlings with extreme

leaf-roll sensitivity, for with proper methods of evaluation such seedlings would be found among existing progenies bred for other purposes at Canberra. Accordingly 155 seedlings were selected at random from 8 complex crossbred progenies involving a wide range of varieties as parents. The seedlings were sprout inoculated in the spring of the 1947-48 season, and then planted at the Dickson Experiment Station. At flowering it was impossible, because of a severe attack of early blight (*Alternaria solani*), to classify the seedlings on their external symptoms. Basal stem sections were examined for the occurrence of phloem necrosis and according to the amount present the seedlings were grouped in Table 10 into three classes: severe, mild, and non-necrotic. Seedlings intermediate between the severe and mild classes were added to the more appropriate class. Tuber samples were kept from the 19 seedlings with a severe necrotic reaction in the phloem.

TABLE 10

PERCENTAGES OF SEEDLINGS CLASSIFIED, AFTER SPROUT INOCULATION, INTO GROUPS ACCORDING TO THE AMOUNT OF PHLOEM NECROSIS PRESENT AT THE FLOWERING STAGE

Number of Cross	Percentage of Seedlings in 3 Classes according to Amount of Phloem Necrosis			Total Number Seedlings
	Severe (50-70%)	Mild (1-20%)	Absent (no necrosis)	
46	5.1	92.3	2.6	39
54	15.4	69.2	15.4	13
67	0.0	100.0	0.0	5
74	28.5	28.5	43.0	7
75	0.0	83.3	16.7	12
76	16.7	72.2	11.1	36
77	14.3	80.9	4.8	21
78	18.2	50.0	31.8	22

Table 10 shows that the percentage of severe phloem reactors among the various progenies is of a low order and that the highest percentages of seedlings occurred in the mild phloem necrotic group. In order to find whether any of the seedlings in the severe group of Table 10 had extreme sensitivity to leaf roll, 3 tubers of each seedling from the field samples were planted in the greenhouse in the winter. Eight weeks from emergence it was found that the results from the 3 tubers of each seedling were in agreement and that 4 of the seedlings had indefinite symptoms (Plate 2, Fig. 1), 4 had medium but well-defined symptoms (Plate 2, Fig. 2), 5 had severe symptoms (Plate 2, Fig. 3), 3 developed into small weak plants (Plate 2, Fig. 4), and 3 did not emerge at all. When the 3 tubers of each of the 3 seedlings which did not emerge were dug up it was found that very small, browned "hair" sprouts incapable of forming plants had developed at some of the "eyes." When these tubers were sectioned the whole of the vascular tissue was found to be brown and necrotic. Basal stem sections of all of the plants with well-developed stems in the groups with indefinite, medium, and severe symptoms were examined for phloem necrosis, which was

found to vary from 30 to 70 per cent., those with the severe symptoms having the highest percentage. Two of the seedlings with indefinite leaf-roll symptoms had 70 per cent. phloem necrosis.

These results made it probable that extreme sensitivity to leaf roll could be developed and that a few seedlings among most progenies had the requisite physiological reactions. It seemed that a rapid and severe leaf-roll reaction, both external and internal, following sprout inoculation, was a desirable basis for the selection of seedlings for further work on this aspect. In order to select additional seedlings for the work on leaf-roll sensitivity, tubers of a further 53 which had been selected in the field because of good agronomic characters were sprout inoculated and planted in duplicate in the greenhouse in the early summer of 1948. A month from emergence the seedlings were classified into severe, medium, and indefinite groups according to the intensity of their external symptoms, and then basal stem sections of each were examined for the occurrence of phloem necrosis. Table 11 summarizes the results obtained in this experiment.

TABLE 11

FIFTY-THREE POTATO SEEDLINGS CLASSIFIED ON EXTERNAL SYMPTOMS AND EXTENT OF PHLOEM NECROSIS ONE MONTH AFTER SPROUT INOCULATION OF VIRUS-FREE TUBERS WITH LEAF ROLL

External Symptoms	Extent of Phloem Necrosis*				Total Number
	None	1-10%	20-40%	60-80%	
Severe	0.0	50.0	21.4	28.6	14
Medium	15.8	68.4	10.5	5.3	19
Indefinite	55.0	40.0	5.0	0.0	20

* Results from the duplicates of each seedling were in good agreement.

Table 11 shows a correlation between external and internal leaf-roll symptoms, owing, no doubt, to the more standardized conditions in the greenhouse. As with Tables 9 and 10, the tendency in Table 11 is for the highest percentage of seedlings to have a light to medium leaf-roll reaction. Only the 4 seedlings giving severe external symptoms together with 60 to 80 per cent. of phloem necrosis have been retained for crossing and further experiment. It is apparent that unless large progenies are investigated, the number of seedlings with the necessary physiological reaction to leaf roll will be limited. In the studies described, limitation of seedlings for leaf-roll sensitivity studies was considerable, as only seedlings with resistance to two other viruses and with desirable agronomic characters were considered. This ensures that when seedlings with leaf-roll sensitivity and perhaps with field resistance to this disease are developed, they will possess some of the other important attributes desired.

VII. DISCUSSION

Owing to a lack of suitable indicator plants and well-defined methods of diagnosis, the general relationship between the leaf-roll virus and the potato plant is not as well known as that existing between this host and some of the other

viruses. As a result, the problem of field resistance of the potato to leaf roll has not been resolved. It is hoped that a study of phloem necrosis, both qualitatively and quantitatively, together with the employment of recently described indicator plants (Kirkpatrick 1948), will facilitate the development of leaf-roll-resistant potato hybrids. Although indicator plants like *Physalis angulata* do not always become easily infected with leaf roll by aphid transmission, stem sections of affected plants show extensive necrosis of the phloem when stained by the phloroglucinol-acid technique. If leaf-roll strains exist, as suggested by the results with the variety Bismark, this phloem reaction in an indicator plant could be useful in distinguishing such strains in varieties and hybrids. The problem of leaf-roll resistance would be complicated considerably if the existence of strains of the virus were proven. In addition, the results presented in this paper could lose some of their significance, whilst some of the inconsistencies would be explained. Until more is known about the leaf-roll virus itself, the selection from hybrid potato progenies of the severe reactors as described seems to offer the best solution to the resistance problem. Severe reactors, both internally and externally, appear to have comparative stability of response to leaf roll under varying environmental conditions. Results to date have encouraged the view that it should be possible to develop almost hypersensitive hybrids. The problem of linking sensitivity with field resistance should not be difficult, as varieties like Arran Crest with this type of combination are already in existence. Even if hypersensitivity to the leaf-roll virus is not achieved, a proportion of the new leaf-roll-sensitive varieties developed will have as great a degree of field resistance as existing varieties, but will cause few difficulties in certification schemes as masking of symptoms will rarely occur under field conditions.

VIII. ACKNOWLEDGMENTS

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EXPLANATION OF PLATES 1-2

PLATE 1

Fig. 1.—A photomicrograph of a section from the base of the stem of a leaf-roll-infected Katahdin plant, showing the stained areas in the external primary phloem.

Fig. 2.—Plants from the two halves of a leaf-roll-infected Bismark tuber, grown in sand (left) and in rich potting loam (right).

PLATE 2

Fig. 1.—Leaf-roll-infected potato seedling with indefinite symptoms.

Fig. 2.—Leaf-roll-infected potato seedling with medium but well-defined symptoms.

Fig. 3.—Leaf-roll-infected potato seedling with severe symptoms.

Fig. 4.—Marked reduction in vigour of a seedling after a season's infection with leaf roll.

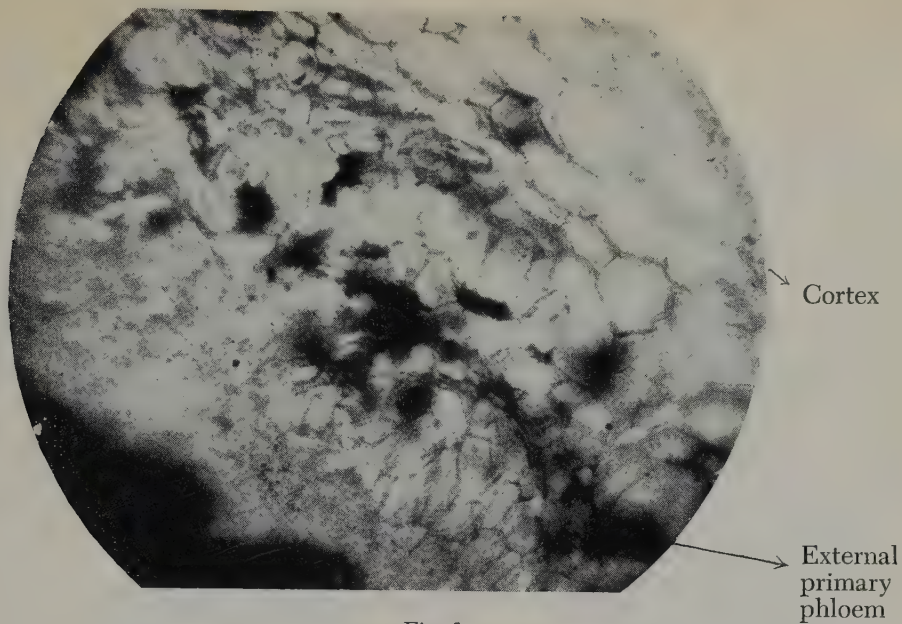


Fig. 1



Fig. 2

HUTTON,—THE SIGNIFICANCE OF THE NECROTIC PHLOEM REACTION IN THE POTATO TO THE LEAF-ROLL VIRUS



HUTTON.—THE SIGNIFICANCE OF THE NECROTIC PHLOEM REACTION IN THE POTATO TO THE LEAF-ROLL VIRUS

ELECTRON MICROSCOPIC STUDIES OF SPERMATOOA

I. THE MORPHOLOGY OF THE SPERMATOZOON OF THE COMMON DOMESTIC FOWL (*GALLUS DOMESTICUS*)

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(Plates 1-11)

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Summary

The morphology of the fowl spermatozoon, as revealed by use of the electron microscope and such techniques as partial enzymic digestion and disruption with distilled water, is described in detail, and compared with that observable by light microscopy.

The sperm head carries at its anterior extremity a spindle-shaped body, the apical spine, which normally is closely covered by a conical membranous cap. The apical cap, which has been overlooked by previous workers, may be detached by dilution of the semen with distilled water. It seems likely that these structures are intimately concerned in the penetration of the vitelline membrane during fertilization.

The axial filament, which contains nine L fibrils arising from the anterior distal centriole and two M fibrils, passes through the mid-piece and continues the full length of the tail, approaching 100 μ in length. It is surrounded in the mid-piece by a number of granules, presumably of mitochondrial origin, which are arranged to give an appearance of bilaterally symmetrical segmentation. The mid-piece is externally surrounded by a delicate membrane easily disrupted in distilled water. There is no evidence for the presence of a spireme or other helically-wound structure in the mid-piece.

In the tail, the axial filament is encased in an amorphous sheath, which decreases in thickness towards the tip of the tail and is easily disrupted by distilled water, allowing the axial filament to fray into eleven fibrils. Two of these fibrils are differentiated from the remaining nine by their dimensions and greater susceptibility to distilled water. It is suggested that the nine L fibrils constitute the locomotor organ of the sperm. It is possible that the two M fibrils function as a rudimentary nervous system.

In direct contrast with the state prevailing in mammalian sperm, there is no helically-wound cord surrounding the axial filament in the tail. This seems to explain why the tails of fowl and of certain other sperm fray easily in distilled water, while those of mammalian sperm do not.

Certain dilution phenomena are explained by the presence of an adsorbed layer of colloidal material, which is removable by great dilution or repeated washing of the sperm. The layer greatly modifies the rate of osmosis in hypotonic solutions. There is no trace of a lipoid or other capsule external to the cell-wall, as has been postulated to explain similar protective phenomena occurring with other sperm.

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I. INTRODUCTION

In order to present a clear picture of the finer detail revealed by electron microscopy, the results previously obtained by conventional microscopic methods will first be summarized.

II. LIGHT MICROSCOPY

Ballowitz (1888) divided the sperm for descriptive purposes into three regions, viz. the head, mid-piece, and tail, and this convention is adhered to in this paper. The head was described as a long cylindrical body, tapering slightly towards the anterior end, and terminating in a small, pointed, and intensely-staining apex. The intensely-staining apex of avian sperm has been described by Schweigger Seidel (1865), Jensen (1886), Brunn (1884), and Ballowitz (1888), the latter author also noting that a few spermatozoa from the semen of certain avian genera ended anteriorly in a very fine point. However, none of these authors realized that this deeply-staining apex is in reality a composite structure, as will be demonstrated later.

According to Retzius (1909) the sperm head is slightly curved helically, but not to the same extent as many other avian sperm, and the resultant hydrodynamic asymmetry is responsible for rotation of the sperm about its horizontal axis during active movement.

Ballowitz (1888) stated that a fine dark stripe could occasionally be observed in the long axis of the head. We have also observed this stripe in specimens stained with iron haematoxylin, but only in sperm with swollen heads, and never in normally-shaped mature sperm. Adamstone and Card (1934) described a series of dark lipoidal granules distributed along the sperm head.

The proximal centriole is a deeply-staining body situated at the posterior extremity of the head (see Fig. 1) and is more clearly visible if the membrane surrounding the mid-piece is removed by maceration with distilled water. It is then seen to be crescent-shaped. The apparently spherical anterior distal centriole lies just behind the proximal centriole, and is separated from it by a short non-staining region, the neck. The axial filament of the tail is derived by growth from the anterior distal centriole.

Ballowitz (1888) claimed to have seen a spirally-twisted refractile cord in the mid-piece of fresh sperm. If the sperm were slightly macerated in salt solutions prior to staining with osmium rosaniline, this spiral could be seen to have six twists around the axial filament. However, this internal structure was not visible unless the sperms were first macerated. Brunn (1884) found that the mid-piece was developed from a number of granules around the axial filament. In the mature sperm these granules were so arranged as to give an appearance of cross-striation. He considered that there was no spiral structure. As will be demonstrated later, the electron microscopical evidence seems to favour Brunn's interpretation of the mid-piece as a segmental structure.

According to Ballowitz (1888), the mid-piece is surrounded by a delicate membrane, continuous with that surrounding the head, and having no observable

segmentation or spiral structure. At the junction of the tail and the mid-piece, and surrounding the axial filament, is the ring-shaped posterior distal centriole.

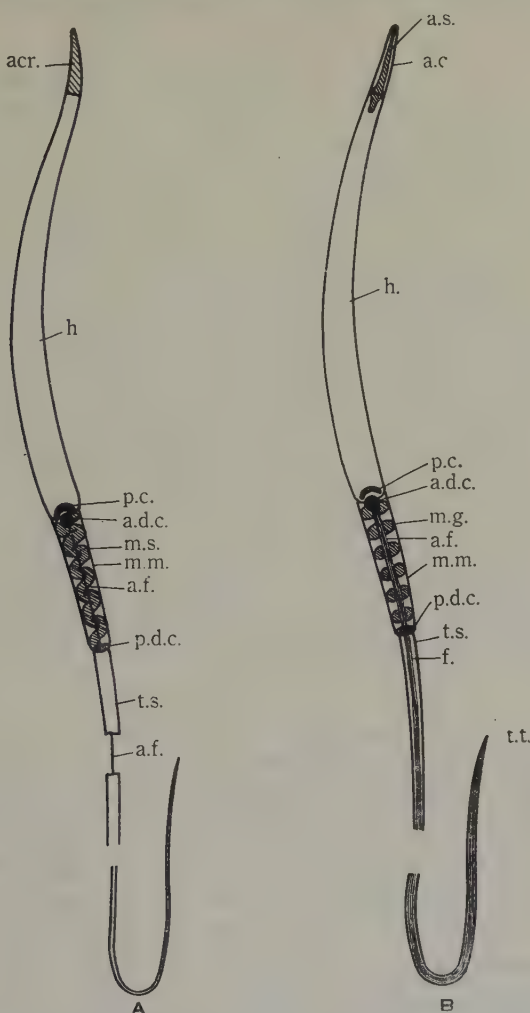


Fig. 1.—Diagrammatic representation of sperm morphology (not to scale). A, Structural features as described by Ballowitz (1888); B, Structural features as determined by the electron microscope.

Acr., acrosome; a.c., apical cap; a.s., apical spine; h., head; a.d.c., anterior distal centriole; a.f., axial filament; m.s., mitochondrial spiral; m.m., mid-piece membrane; p.d.c., posterior distal centriole; t.s., tail sheath; p.c., proximal centriole; m.g., mitochondrial granules; f., fibrils in axial filament; t.t., tapering tip of tail.

The tail proper is about two-thirds the diameter of the mid-piece at its junction with the latter, and tapers gradually to terminate in a sharp point

without any definite structural changes. Schweigger Seidel (1865) working on the sperm of the finch noted that the sperm tail contained a number of fibrils. Ballowitz (1888) found that the axial filament in finch sperm could be separated by maceration in glycerin into 2, 7, 10, or 11 fibrils, and that the axial filament in fowl sperm, which was enclosed by a sheath varying in thickness along the length of the tail, contained at least 7 fibrils.

We have observed that if fresh fowl semen is diluted about one hundred times with distilled water, the tail sheath, which is normally deeply stained by iron haematoxylin, is often destroyed. If the water-macerated wet smear is fixed in formalin, dried over a flame, and stained with iron haematoxylin, the lightly-staining axial filament can occasionally be seen to have separated into several fibrils. However, use of the electron microscope has shown that the tail fibrils are well below the resolution limit of the light microscope, and are visible optically only by virtue of their length and the diffraction effect which is commonly observed with fibrils of diameters below the limit of resolution. It is therefore all the more remarkable that Ballowitz was able to note seven fibrils in the fowl sperm tail and eleven fibrils in the finch sperm tail.

Recently there have been a number of electron microscopical studies of sperm by Baylor, Nalbandov, and Clark (1943), Harvey and Anderson (1943), Bretschneider and van Iterson (1947), and Seymour and Benmosche (1941). It has been shown that the tails of human, bull, and *Arbacia* sperm contain about ten fibrils.

III. MATERIALS AND METHODS

Samples of semen were collected from Rhode Island Red and White Leghorn males using the method of abdominal massage described by Burrows and Quinn (1937). Before preparing mounts for examination in the electron microscope, it was found necessary to isolate the spermatozoa from the colloidal and other material present in the semen, without damaging any delicate structure in the sperm. These conditions were realized by application of the following procedure.

Samples of fresh semen were diluted with 10 to 20 volumes of Tyrode solution. The sperm were freed from colloidal material by repeatedly spinning them down in the angle centrifuge at 3000 r.p.m. and resuspending the sediment in fresh Tyrode solution, the supernatant fluid being discarded each time. Motility was unimpaired by the dilution with Tyrode solution and there was no apparent damage as a result of centrifugation, since the motility was still very high after as many as five centrifugations. In general, it was sufficient to spin the sperm down three times in Tyrode solution to free them from extraneous material. The sperm were then fixed by the addition of a small volume of 10 per cent. formalin. Motility ceased immediately, and the formalin-Tyrode suspensions were kept as stock at 0°C. It should be noted that prior to fixation the sperm were actively motile, indicating that they were still functionally intact, at least with respect to their motility. Examination with

the light microscope showed no difference between sperm washed in Tyrode solution and those in the fresh undiluted semen. It has been our general experience in this Laboratory that formalin is the most reliable fixative for electron microscopic work, and we have never observed any artifact or distortion attributable to its use.

After at least 24 hours standing in contact with the Tyrode-formalin solution, the sperm were spun down and washed several times by centrifugation in distilled water. The final suspension in distilled water was ready for mounting on the conventional electron microscope specimen screens. In most cases the mounts were prepared either by allowing a drop of the suspension to dry on the specimen screen (i.e. on a collodion film supported by the screen), or by removing excess fluid with filter paper. It is worthy of note that the formalin-fixed sperm were immune to the osmotic effects of distilled water (cf. preparations made when the semen was initially diluted with distilled water), the centrifugation merely serving the purpose of freeing the sperm from the salts present in Tyrode solution. As a result, a salt-free suspension was obtained from which it was convenient to prepare specimens for examination. When suspensions in Tyrode solution are dried, there is a possibility of serious distortion of the sperm due to crystallization of salts, even though these may be subsequently washed from the screens with distilled water. Electron micrographs of specimens prepared by the technique described showed that all parts of the sperm were intact and free from distortion but gave little information concerning the internal structures.

The effects of distilled water on unfixed sperm were in striking contrast to its lack of effect on fixed sperm, and permitted much of the structure to be determined. These effects in general were due to the disruption caused by osmosis, and were most marked when sperm washed by centrifugation in Tyrode solution were resuspended in distilled water. Motility ceased immediately, and was lost irreversibly. The effects of such treatment, as seen in electron micrographs, will be considered in detail later. It will be apparent that the loss of motility is due to disruption and fraying of the tail together with damage to the motor region of the sperm.

Dilution of fresh semen with small amounts of distilled water did not immediately produce such marked disruption, and motility persisted in some degree for several hours. However, repeated centrifugation in distilled water rapidly resulted in the loss of all motility. The relative slowness of the osmotic effects on spermatozoa in semen when moderately diluted with distilled water seems to indicate that colloidal material present in the semen, some of which is adsorbed on the sperm, tends to act as a protective "blanket" in preventing rapid osmosis.

To check any possible distortions or artifacts introduced by the methods of specimen preparation, the Altmann-Gersh technique of freeze-drying, in which all structures are dimensionally preserved, was used. However, the method was not very suitable for fowl spermatozoa, since, as a consequence of their great length, they tended to form a three-dimensional network and did

not lie flat on the collodion film. Although the authors were able to show that the frozen-dried sperm did not differ appreciably in appearance from those dried in air after the Tyrode-formalin treatment already described, the quality of the micrographs was poor. The dimensions of the frozen-dried sperm indicated that a moderate amount of shrinkage in all dimensions occurred in air-dried specimens. Dawson and MacFarlane (1948) and Farrant and O'Connor (1949) have discussed the alterations in dimensions of viruses, when dried in air, for both fixed and unfixed specimens. Their results appear to be generally applicable to biological objects.

In most instances it was found desirable to increase the image contrast by employing "electron stains." These reagents, of which phosphotungstic and phosphomolybdic acids are those generally employed, combine with the specimen material and increase its effective electron scattering power by virtue of the elements of high atomic number contained within them. Staining was carried out by placing a drop of 0.1 per cent. solution of the reagent on the freshly-dried specimens already mounted on the screens, leaving it for a minute or two, and washing off the excess with distilled water. The technique has been of great value in biological work (Hall, Jakus, and Schmitt 1945).

In addition to the methods already outlined, the technique of shadow casting, first described by Williams and Wyckoff (1946), was generally applied. The deposition of such thin films of heavy metals increases the contrast and definition of the image, and also permits surface structures on relatively thin objects to be examined in greater detail, since the greater part of the electron scattering power of the specimen is then concentrated in the metal film. The heavy metal used was platinum, which, although more difficult to evaporate than gold, does not migrate and coagulate under the influence of the electron beam (Mandle 1947). While using a cathode-biased electron gun we have found it impossible to use gold with any degree of success at the high beam intensities required for accurate focusing.

A technique which has been of great assistance in the elucidation of the internal structure of the fowl spermatozoon is that of partial digestion with various enzymes. Good results were obtained by digestion with pepsin for about 30 minutes at pH 3 and 37°C. For this purpose formalin-fixed sperm were used. Suspensions of spermatozoa in Tyrode-formalin solution were washed several times by centrifugation in distilled water, acidified to pH 3, and a small crystal of pepsin added, before incubation at 37°C.

Acidified suspensions without the enzymes were used as controls, and electron micrographs showed that these were intact and indistinguishable from untreated sperm at the end of the incubation period. Similar techniques were employed in carrying out tryptic digestion* in buffer solutions at pH 8.5.

It should be realized, when using formalin-fixed preparations of sperm to study the effects of enzymic attack on the morphological structures therein, that the results may be quite different from those obtained when unfixed dead

* The enzymes used in the investigation were "Difco" pepsin and "B.D.H." trypsin.

sperm are used. Our choice of formalin-fixed sperm as material on which to carry out such enzymic digestions was occasioned solely by the wealth of morphological detail revealed.

The electron microscope used was an RCA Model EMU, fitted with biased electron gun and objective aperture diaphragm. Fifty-kilovolt electrons were used throughout the investigation. The instrument was calibrated for magnification by the method of Farrant and Hodge (1948), in which glass fibres are calibrated interferometrically and used as standard objects in the electron microscope. The method permits the magnification to be determined to within 1 per cent.

IV. MORPHOLOGY

Plate 1, Figures 1 and 2, illustrates the appearance of sperm after being washed in Tyrode solution and fixed by the addition of formalin. The general outline is essentially similar to that observed by light microscopy in fresh semen and stained smears. A consideration of shadow lengths in Plate 1, Figure 2, makes it clear that the sperm head has maintained a roughly circular cross-section (i.e. only a small amount of flattening and collapse has occurred) and that a moderate amount of flattening (about 30 per cent.) has occurred in the mid-piece and tail, in accordance with the higher degree of hydration to be expected in the latter structures. The ratio of shadow length to object height is approximately 4:1 in the electron micrographs of shadow-cast specimens. It seems reasonable to claim that Plate 1, Figures 1 and 2, is both dimensionally and in appearance a close representation of the living sperm, provided that due allowance is made for the inevitable dehydration of the specimen in electron microscopic examination.

(a) *General Appearance*

The sperm head is an elongated cylinder, about 14μ in length and not exceeding 0.5μ in diameter, tapering slightly towards the anterior extremity, where a distinct differentiated structure may be seen, viz. the apical cap. The complete opacity of the heads to 50-kv. electrons indicates that the intracellular material of the head is of relatively high mass density, and is in agreement with the belief that this material is mainly nuclear in a highly condensed form.

Immediately following the head is the mid-piece of the sperm, averaging about 4μ in length and 0.5μ in diameter. Very little internal structure other than a vague appearance of segmentation can be discerned in the electron micrographs of sperm washed in Tyrode solution and fixed in formalin (Plate 1, Figs. 1 and 2).

The anterior extremity of the tail proper is marked by a sudden discontinuity in outline (Plate 3, Fig. 3) corresponding to the position of the posterior distal centriole. Proceeding posteriorly from the tail mid-piece junction, the tail presents a smooth appearance and narrows slowly without any discontinuity of outline until a point about 2μ from the extreme tip is reached. At this

point the outline narrows more abruptly and the tail ends in a fine pointed tip (Plate 1, Fig. 1). The discontinuity of outline is not, however, as abrupt as in mammalian sperm. The last 2μ of tail appears to be devoid of sheath. The overall length of fowl spermatozoa generally exceeds 100μ .

(b) Detailed Morphology

(i) *The Sperm Head*.—Plate 2, Figure 1, illustrates the effect on the fresh sperm of washing by centrifugation in distilled water. The apical caps of the sperm are more easily visible and many are completely detached (Plate 3, Fig. 1). Detachment of the apical cap from the head exposes a spear-like structure, these two structures together constituting the acrosome (Plate 2, Figs. 2 and 3). These apical spines are readily observable with the light microscope and are presumably identical with those described by Ballowitz (1888), who did not, however, describe the apical caps. Plate 2, Figure 4, shows a cap partly detached from a sperm head, the apical spine of which may be faintly distinguished. The electron micrographs make it clear that these caps are conical in shape and fit snugly over the apical spine in the intact sperm. They appear to be moderately susceptible to pepsin (Plate 8, Fig. 2). The authors are not aware of any previous differentiation of the acrosome into these two structures.

In specimens prepared from fresh semen by dilution and centrifugation in distilled water, the number of sperm with spines exposed by detachment of the apical caps generally exceeded the number of intact sperm. However, when the sperm were washed by repeated centrifugation in Tyrode solution, and the residue resuspended in distilled water, almost all the sperm were affected, the caps being detached and the spines exposed (Plate 4, Fig. 2, and Plate 5). This result is in agreement with the greater "osmotic shock" produced by the latter procedure, as indicated by the more rapid loss of motility and greater disruption of the mid-piece and tail.

While digestion with pepsin had little effect on the sperm head, prolonged incubation at pH 8.5 with trypsin produced the effects shown in Plate 10, Figure 3, and Plate 11, Figure 1. The cell-wall was removed, allowing the contents of the head to expand and spread on the mount, where they were partially digested while the apical spine and cap remained unaffected. It will be clear from a consideration of Plate 2, Figures 2 and 4, Plate 3, Figure 1, Plate 10, Figure 3, and Plate 11, Figure 1, that the apical spine is a spindle-shaped body about $1.3\text{--}1.5\mu$ long and 0.1μ in diameter at maximum cross-section; its posterior part is embedded in the sperm head, the anterior part normally covered by the conical cap. It is partially damaged by pepsin and unaffected by trypsin.

The fine strands of densely-staining material to be seen within the sperm head in Plate 11, Figure 1, presumably represent the remains of the chromatin network of chromosomes in the resting phase, after the breakdown of interstitial material and cell-wall by the tryptic digestion.

(ii) *Mid-piece and Tail*.—It will be convenient to consider the structure of the mid-piece and tail regions together, since they are both traversed by the axial filament or central core, which is in itself a composite structure consisting of a number of fibrils.

The mid-piece is bounded anteriorly by the anterior distal centriole, and posteriorly by the posterior distal centriole (see Plate 3, Fig. 3, Plate 4, Fig. 1, Plate 11, Fig. 2). In sperm washed in Tyrode solution and fixed in formalin (Plate 1, Figs. 1 and 2) the mid-piece does not exhibit much internal structure other than a vague segmentation. Traces of an outer limiting membrane are occasionally visible (Plate 1, Fig. 2). Much more of the structure may be determined by examination of sperm treated with distilled water. The thin outer membrane is disrupted and a number of granules are liberated. Some detached granules are visible in Plate 4, Figure 2, and Plate 5. For the most part, however, the granules, which are themselves swollen and broken down to some extent by distilled water (Plate 4, Fig. 1), adhere to the axial filament and appear to be arranged in a fairly regular manner to give an appearance of segmentation (Plate 4, Fig. 2, Plate 11, Fig. 2). Plate 11, Figure 2, shows that the arrangement of the granules is symmetrical with respect to the axis of the sperm. These granules are probably of mitochondrial origin, and while the electron microscopic evidence seems to favour the segmental type of arrangement proposed by Brunn (1884) for the mid-piece rather than the spiral arrangement described by Ballowitz (1888), it does not preclude the possibility that a spireme exists in the intact sperm. Indeed, such a segmental arrangement contrasts with the definite helical cord to be found in the mid-piece of certain mammalian sperm.

The posterior distal centriole was often visible as a marked discontinuity in the outline of the sperm, lying at the posterior extremity of the mid-piece and the beginning of the tail (Plate 3, Fig. 3). Its appearance in electron micrographs confirms the observation of Ballowitz (1888), who described it as an annular body surrounding the axial filament.

The tail region of the sperm comprises the axial filament surrounded by an apparently amorphous membranous sheath (Plate 3, Fig. 2). In the region immediately following the posterior distal centriole the sheath is comparatively thick, but gradually tapers off towards the tip of the tail and ends about 2μ from the tip, as seen in sperm washed in Tyrode solution and fixed in formalin. However, the tail sheath does not end so abruptly as in the case of bull sperm (Bretschneider and van Iterson 1947). Since the apparently naked tip does not fray into fibrils when fixed sperm are examined, it seems possible that the axial filament is itself surrounded by an extremely thin and delicate membrane, which is in turn surrounded by the tail sheath proper. We have not, however, observed such a membrane directly. The phenomenon could also be due to the presence of the matrix material, in which the fibrils are set.

When fresh sperm were washed in Tyrode solution and resuspended in distilled water, marked disruption of the tail occurred, as shown by fraying of the axial filament into fibrils and detachment of portions of the tail sheath.

However, fraying of the tail always occurred in the distal regions and was never observed to occur closer than 30 or 40 μ to the mid-piece (Plate 5), presumably because of the relative stoutness of the tail sheath in this region. Detached portions of the tail sheath are shown in Plate 6, Figures 1 and 2. The sheath material is apparently amorphous and there are no helically-wound fibrils such as were found in bull sperm by Bretschneider and van Iterson (1947). This explains the ease with which the tails of fowl sperm are disrupted by distilled water. The difficulty of fraying tails of bull sperm may be ascribed to the presence of such a helically-wound binding, which would necessarily be more difficult to disrupt.

The structure of the axial filament has been demonstrated by examination of sperm treated with distilled water, which results in fraying into a number of well-defined fibrils (Plate 6, Fig. 3, Plate 7, Figs. 1 and 2). Careful counting of the fibrils from single sperm tails has shown that the total number is eleven, nine of which are remarkably uniform in diameter and appearance and relatively insusceptible to the action of distilled water, their average diameter being about 400 Å. If we allow for dehydration and flattening during drying, it seems reasonable to estimate that the fibrils in the living sperm are approximately circular in cross-section and about 450 Å in diameter. The remaining two fibrils of the eleven are obviously differentiated from the other nine (Plate 7, Figs. 1 and 2) and much more susceptible to the destructive action of distilled water, making it difficult to obtain an accurate estimate of their diameter. In the absence of further criteria for distinguishing between the two types of fibrils, we propose, for the purposes of the present paper, to call the latter two fibrils the M fibrils, and the remaining nine the L fibrils.

It will be evident from Plate 7, Figure 2, that the fibrils have fractured in several places as a result of the stresses encountered during the final stages of drying, and further, that the broken ends are connected by thin strands of material. It seems likely that the fibrils are composite structures consisting of a central elastic core surrounded by a relatively inelastic material, or the thin strands may merely represent portions of the amorphous matrix material adhering to the surfaces of the fibrils (see Plate 6, Fig. 3). It is significant that in trypsin-treated specimens there is an abundance of fine fibrils about 50-100 Å in diameter, similar to those shown in Plate 11, Figure 1. These may well represent the thin elastic cores of the tail fibrils after removal of the external material by tryptic digestion. Apart from this apparent complexity of structure of the tail fibrils, they appear to be distinct entities, and we have not observed any such breakdown into subfibrils as was claimed by Bretschneider and van Iterson (1947) in the case of bull sperm.

While we have not observed any regular structure on a small scale in the tail fibrils, it will be obvious from the micrographs that there is some fine structure present. In particular, there is in Plate 6, Figure 3, a fine periodicity reminiscent of that observed in clam muscle by Hall, Jakus, and Schmitt (1945). That this periodicity is somewhat ill-defined and irregular is probably due to the distorting action of the distilled water, but it seems to lie in the range 150-200 Å.

The axial filament, consisting of a number of fibrils, arises from the anterior distal centriole, passes through the annular posterior distal centriole, and extends the full length of the tail, maintaining its fibrillar nature throughout. It is believed that the axial filament develops from the anterior distal centriole during spermatogenesis. Although our observations were of mature sperm, the structures revealed by the electron microscope are in agreement with this hypothesis (Plate 4, Fig. 1, Plate 9, Fig. 1, Plate 10, Fig. 2). The anterior distal centriole, which is clearly seen in pepsin-treated sperm, appears to be a composite body, and it is our belief that each fibril of the axial filament develops separately from one of a number of small bodies which together comprise the anterior distal centriole. The relation of the fibrils to the anterior distal centriole and the sperm head will be evident from a consideration of Plate 8, Figure 2, and Plate 10, Figure 2. The concavity in the sperm head in Plate 10, Figure 2, presumably represents the posterior aspect of the crescent-shaped hemispherical proximal centriole.

Incubation of formalin-fixed sperm at 37°C. with pepsin at pH 3 for 45 minutes resulted in complete digestion of the tail proper. Plate 8, Figure 2, shows a typical field from such a preparation, while Plate 9, Figures 1 and 2, and Plate 10, Figures 1 and 2, illustrate the process of separation of the anterior distal centriole from the sperm head. It will be seen that those parts of the tail fibrils immediately adjacent to the anterior distal centriole, approximately 4 μ in length and corresponding to that part of the axial filament contained within the mid-piece, are resistant to peptic digestion. There was no trace of those parts of the fibrils contained within the tail sheath or of the sheath itself. This observation suggests that the portion of each fibril contained within the mid-piece is differentiated from the remainder of its length, at least with respect to its resistance to peptic digestion. This may well be related to some functional difference between the two parts.

Another feature of interest in the pepsin-treated sperm is that the number of fibrillar remnants is nine, implying either that the two M fibrils described earlier are not present in the mid-piece, or that they are susceptible to the action of pepsin. In view of the differentiation of the M fibrils with respect to the effects of distilled water, the latter possibility seems more likely.

In samples of sperm subjected to treatment with distilled water, we have often obtained micrographs indicative of a lateral association of the fibrils. Plate 8, Figure 1, illustrates this point. The number of fibrils associated in this manner never exceeded nine, suggesting that only the nine L fibrils are involved in the lateral association to form a sheet-like structure. It seems likely that this sheet of fibrils is rolled up about the long axis to form a tube-like axial filament, the central space of which is occupied by the two M fibrils and cytoplasm. This is supported by the appearance of the axial filament in Plate 10, Figure 1, in which the tube-like structure is clearly visible. Such close lateral association of the fibrils over considerable distances was commonly observed, and could not be attributed to chance aggregation or flattening out of

a homogeneous circular bundle of fibrils. The interpretation of these observations as due to preferential bonding in one plane is not inconsistent with the known properties of proteins. In this connexion, it is of interest to note that a similar lateral association of protein filaments exists in the myofibrils of toad striated muscle (Draper and Hodge 1949).

One further point concerning the structure of the tail is illustrated in Plate 2, Figure 1, and Plate 3, Figure 3. We often observed a cross-striation in the tails of sperm treated with distilled water, particularly in shadow-cast preparations. The same phenomenon was observed by Harvey and Anderson (1943) in the sperm of *Arbacia punctulata*, but no explanation was attempted. The cross-striation may be seen both in single fibrils and in apparently intact tails, the latter implying that the tail fibrils are perfectly aligned transversely to the long axis of the tail. The repetition distance is not very regular and is about 1000 Å. This cross-striation does not appear to be a reproducible feature of the fibrils, and is often not visible at higher magnifications (Plate 6, Fig. 3). The evidence suggests that it may be due to a periodic adhesion or aggregation of some additional material, possibly the amorphous cementing material, external to the fibrils themselves. It cannot be construed as arising from the presence of a helically-wound fibril in the tail sheath. In some respects the striation seems to resemble that observed in paramyosin by Hall, Jakus, and Schmitt (1946).

V. DISCUSSION

Use of the electron microscope in the determination of the morphology of the fowl sperm has resulted in a clear understanding of the structure of a number of morphological features.

The first of these to be considered will be the group of structures comprising the anterior extremity of the sperm head. These are the apical spine and cap. It seems likely that they are intimately concerned with penetration of the vitelline membrane during the process of fertilization of the ovum. Several possibilities suggest themselves in this connexion.

- (a) The apical spine may act as a mechanical agent effecting penetration of the vitelline membrane, the apical cap serving merely as a protective sheath during the passage of the sperm along the oviduct.
- (b) The apical spine may serve a skeletal function in supporting the apical cap until the site of fertilization is reached. The latter perhaps contains enzyme systems capable of producing a localized breakdown of the vitelline membrane.
- (c) Perhaps the most likely mechanism is a combination of (a) and (b), in which enzyme systems of the cap produce localized damage in the vitelline membrane, thus facilitating mechanical penetration by the apical spine.

The complexity of structure in the locomotor regions of the sperm (i.e. mid-piece and tail) is also worthy of discussion. Although there has been in

the past much speculation concerning the mechano-chemical aspects of sperm locomotion, it is apparent that the formulation of satisfactory theories has been handicapped by the lack of precise knowledge of the structures concerned. Although an electron microscopical study such as this cannot pretend to make good all such deficiencies of morphological detail, the finer structural detail revealed by the much greater resolving power should, if used in conjunction with histochemical and physiological data, contribute to the elucidation of the physicochemical processes underlying such biological activities as the locomotion of sperm, protozoa, and bacteria, muscle contraction, and nerve activity.

The axial filament comprises nine L fibrils, laterally associated to form a lamellar sheet folded longitudinally to give an approximately circular cross-section (possibly tubular). These fibrils extend from the anterior distal centriole throughout the length of the mid-piece and tail. In addition, two M fibrils, differing from the L fibrils in their greater susceptibility to distilled water and peptic digestion, run the length of the tail. It is possible that the L fibrils constitute that part of the motor organ of the tail which is responsible for conversion of chemical energy into mechanical motion, and that the M fibrils perform an excitatory function in initiating the activity of this "motor organ." The presence of two such fibrils is interesting in view of the apparent bilateral symmetry of the mid-piece.

The complex structure of the mid-piece, with its differentiated central core of fibrils, layer of mitochondrial granules, and delicate limiting membrane, strongly supports the view that it is the site of the mechanism controlling the activity of the motor fibrils of the sperm tail, the control perhaps being exercised through the medium of the two M fibrils. Determination of the permeability and other characteristics of the membrane surrounding the mid-piece would doubtless be of value in correlating the effects of osmotic and other environmental changes on the motility of the sperm.

The fibrils of the axial filament may be construed as contractile elements analogous to those described in striated muscle by Hall, Jakus, and Schmitt (1946) and Draper and Hodge (1949). There are as yet, however, insufficient data for the formulation of a working hypothesis of the mechanical aspects of sperm locomotion. Further work on the macromolecular arrangement within the fibrils will be useful in determining whether the fibrils may correctly be regarded as contractile elements responsible for sperm locomotion.

The effect of the colloidal matter present in semen in protecting sperm against the osmotic effects of dilution with distilled water is interesting in view of the fact that, in the past, such effects have often been attributed to the presence of a protective lipid capsule, usually assumed to be a morphological feature of the sperm. Our observations have indicated, at least in the case of the fowl sperm, that this is not the case, since we have failed to find any evidence for the presence of any protective capsule external to the cell-wall. The observation that fowl sperm, if suitably washed by repeated centrifugation in Tyrode solution, are extremely sensitive to osmotic shock, indicates that such dilution effects may be attributed to the protection afforded by the layer of

colloidal material adsorbed on the surfaces of the sperm, and that loss of motility may be satisfactorily correlated with the disruption of the delicate membrane surrounding the mid-piece of the sperm. The adsorbed colloidal layer is removed by repeated washing with Tyrode solution.

The theory explains (*a*) why semen may be considerably diluted with distilled water with persistence of some motility for several hours, and (*b*) why sperm washed in Tyrode solution, if exposed to the same dilution in distilled water, immediately lose all motility.

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APPENDIX I

Procedures in treatment of fresh semen prior to mounting sperm on specimen screens were as follows:

- (*a*) Semen diluted with 20 volumes Tyrode solution, washed three times by centrifugation in Tyrode solution, small volume of 10 per cent. formalin added, stored for 24 hours, washed twice by centrifugation in distilled water.
- (*b*) Initial treatment as in (*a*), but no formalin added. Sedimented sperm from Tyrode suspension resuspended in distilled water.
- (*c*) Semen diluted with 20 volumes distilled water, washed three times by centrifugation in distilled water.

- (*d*) Final suspension from treatment (*a*) acidified with HCl to pH 3, pepsin added, incubated at 37°C. for 45 minutes. Reaction stopped with formalin and residue washed twice by centrifugation in distilled water.
- (*e*) Final suspension from treatment (*a*) buffered to pH 8, trypsin added, stored at room temperature for five days.

EXPLANATION OF PLATES 1-11

Acr., acrosome; a.c., apical cap; a.s., apical spine; h., head; a.d.c., anterior distal centriole; a.f., axial filament; m.s., mitochondrial spiral; m.m., mid-piece membrane; p.d.c., posterior distal centriole; t.s., tail sheath; p.c., proximal centriole; m.g. mitochondrial granules; f., fibrils in axial filament; t.t., tapering tip of tail; m., mid-piece; t., tail.

PLATE 1

- Fig. 1.—Treatment (*a*), unstained, showing general outline and appearance of intact sperm.
- Fig. 2.—Treatment (*a*), unstained, shadow-cast with platinum, showing general outline and vertical profile as indicated by shadow edge.

PLATE 2

- Fig. 1.—Treatment (*c*), unstained, shadow-cast with platinum, showing partial disruption of apical cap, mid-piece and tail, periodicity in tail and tail fibrils.
- Fig. 2.—Treatment (*c*), stained with phosphomolybdic acid, showing apical spine exposed by detachment of apical cap.
- Fig. 3.—Treatment (*c*), unstained, shadow-cast with platinum, illustrating vertical profile of sperm head and apical spine.
- Fig. 4.—Treatment (*c*), stained with phosphomolybdic acid, apical cap in process of detachment from head, apical spine partly exposed.

PLATE 3

- Fig. 1.—Treatment (*c*), unstained, shadow-cast with platinum, showing one apical cap detached and one still in position.
- Fig. 2.—Treatment (*c*), unstained, shadow-cast with platinum, showing smooth tail sheath and disruption of mid-piece.
- Fig. 3.—Treatment (*c*), unstained, shadow-cast with platinum, axial filament in mid-piece exposed by removal of mitochondrial granules. Note posterior distal centriole and periodicity in tail fibrils.

PLATE 4

- Fig. 1.—Treatment (*b*), stained with phosphomolybdic acid, showing the axial filament running from the anterior distal centriole through the disrupted mid-piece into the tail sheath.
- Fig. 2.—Treatment (*b*), unstained. Note fraying of the axial filament in the mid-piece region, the apparent segmental arrangement of the granules and the detached apical cap.

PLATE 5

- Treatment (*b*), unstained. Typical field showing exposed apical spines, detached apical caps, disruption of mid-piece, and fraying of tail.

PLATE 6

- Fig. 1.—Treatment (*b*), stained with phosphomolybdic acid. Disrupted and detached portions of tail sheath.
- Fig. 2.—Treatment (*c*), shadow-cast with platinum, showing tail fibrils and detached portion of tail sheath.
- Fig. 3.—Treatment (*c*), stained with phosphomolybdic acid. Axial filament frayed into characteristic fibrils. Note transverse strands of cementing material adhering to fibrils.

PLATE 7

- Fig. 1.—Treatment (*c*), shadow-cast with platinum, showing total of eleven fibrils, two of which are differentiated from the remainder.
- Fig. 2.—Treatment (*c*), shadow-cast with platinum, showing the eleven component fibrils of the tail. Note the two M fibrils and the thin strands connecting the ends of the fibrils after fracture.

PLATE 8

- Fig. 1.—Treatment (*b*), stained with phosphomolybdic acid, showing close lateral association of the L fibrils to form a sheet.
- Fig. 2.—Treatment (*d*), shadow-cast with platinum. Typical field showing remnants of axial filaments attached to heads. Note also effect of pepsin on the acrosome.

PLATE 9

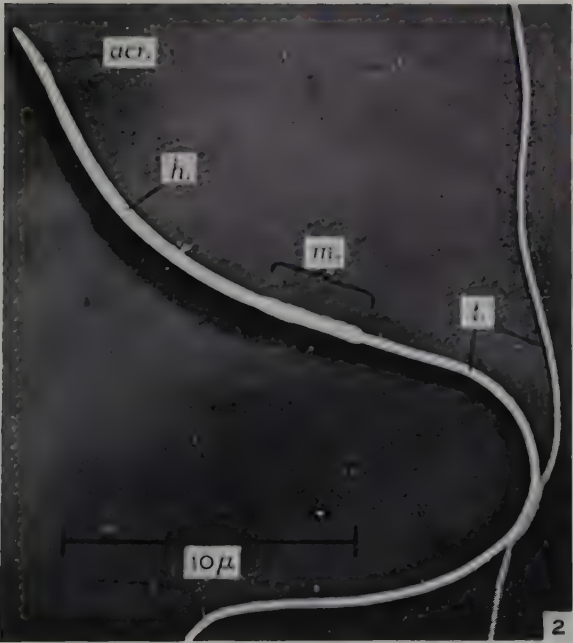
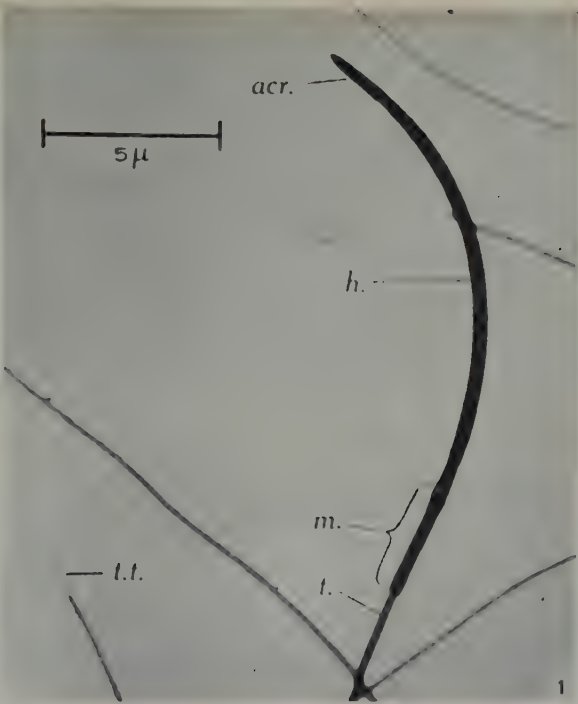
- Fig. 1.—Treatment (*d*), shadow-cast with platinum, showing nine fibrillar remnants arising from distal centriole, which has become detached from the sperm head.
- Fig. 2.—Treatment (*d*), stained with phosphomolybdic acid, illustrating nine fibrils arising from the detached anterior distal centriole. Note pointed ends of the fibrils.

PLATE 10

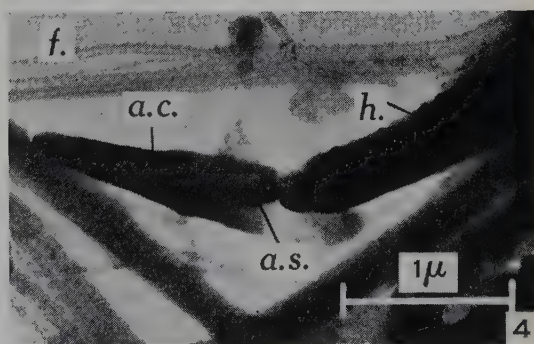
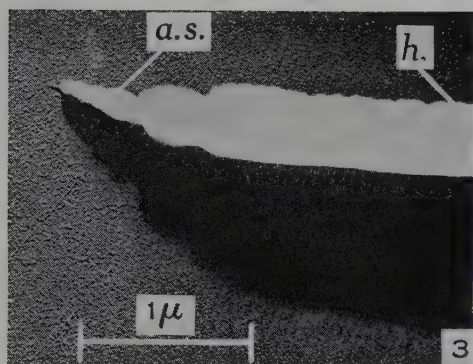
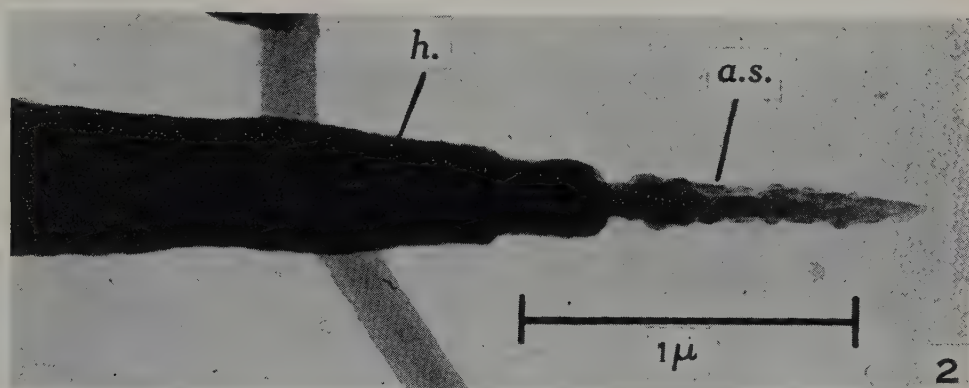
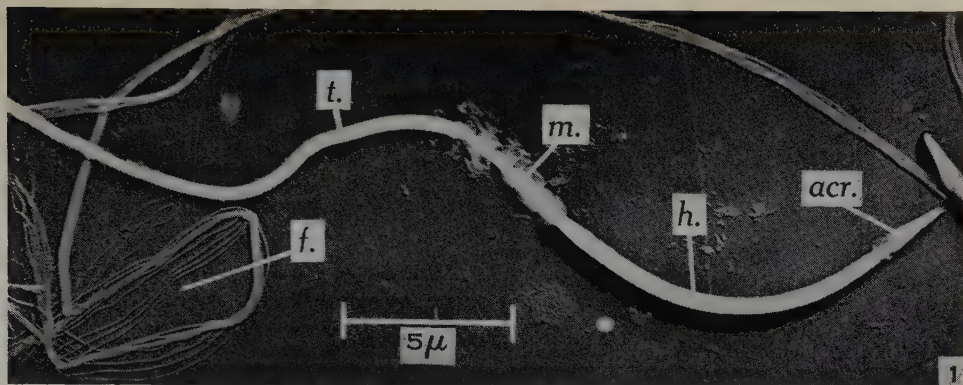
- Fig. 1.—Treatment (*d*), stained with phosphomolybdic acid. Note projections on centriole and folded sheet of fibrils.
- Fig. 2.—Treatment (*d*), stained with phosphomolybdic acid, showing detached centriole and nine fibrillar remnants. Note the concavity in the rear end of the sperm head after detachment of the anterior distal centriole.
- Fig. 3.—Treatment (*e*), stained with phosphomolybdic acid. Typical appearance of sperm after tryptic digestion. Note intact apical cap and spine, disruption of sperm head with release of chromatin material, appearance of anterior distal centriole and remnant of tail.

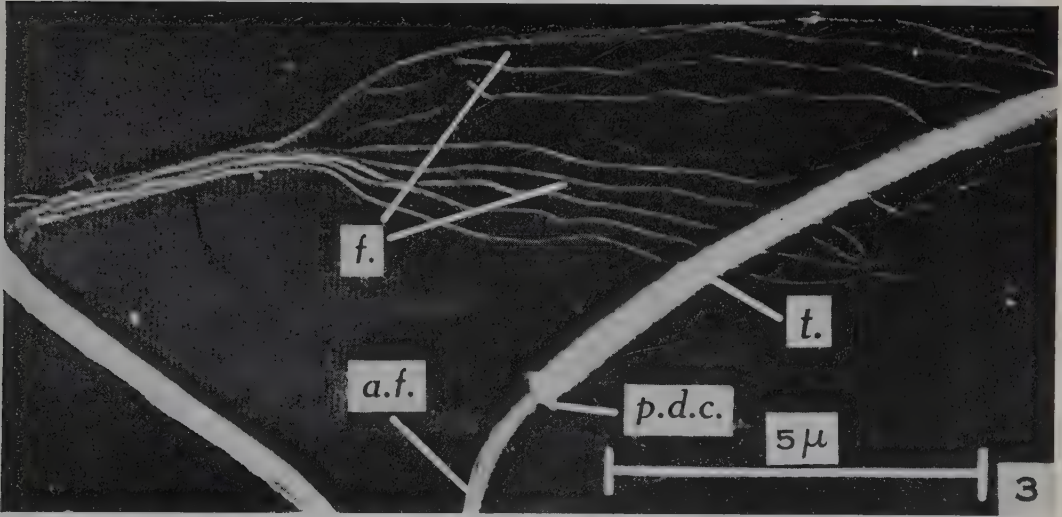
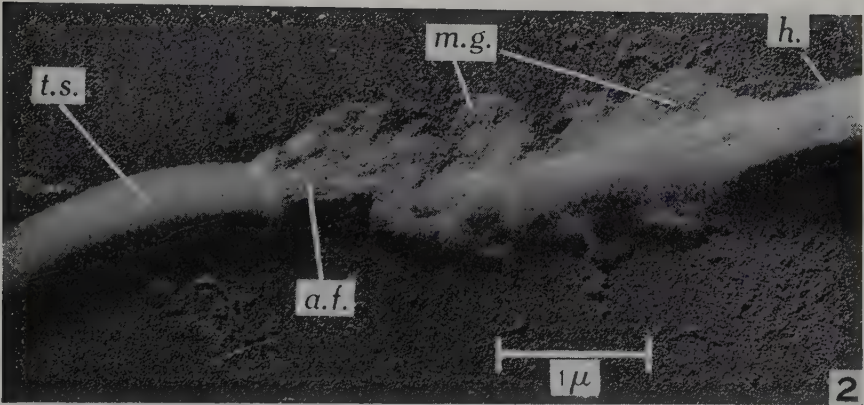
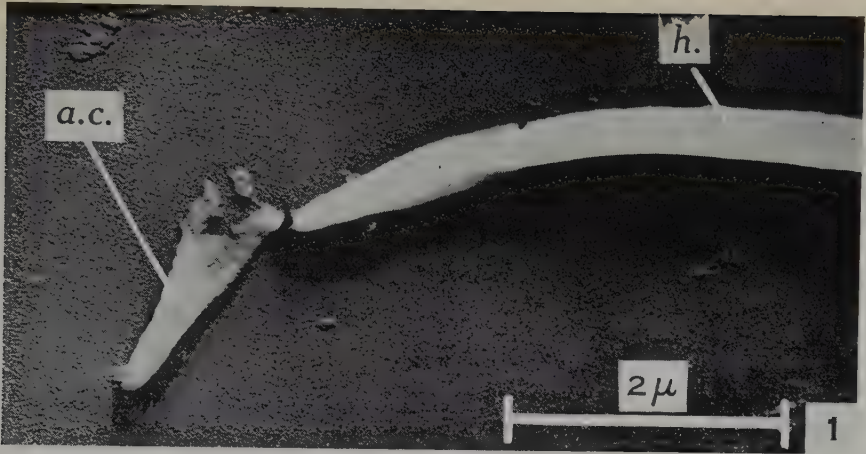
PLATE 11

- Fig. 1.—Enlarged portion of Plate 10, Figure 3. Note structures previously mentioned and very fine fibrils in the background, possibly the cores of L fibrils remaining after tryptic digestion.
- Fig. 2.—Treatment (*e*), shadow-cast with platinum. Mid-piece and tail remnant after tryptic digestion. Note the apparent bilateral symmetry in the arrangement of the mitochondrial granules within the mid-piece.

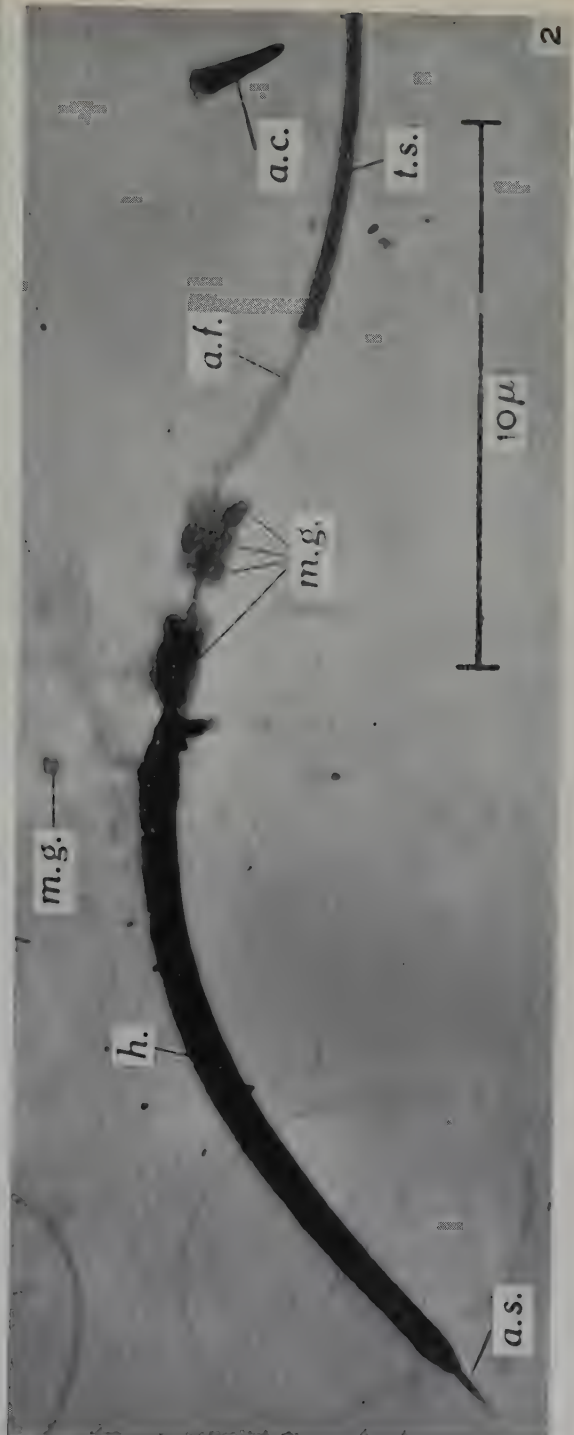
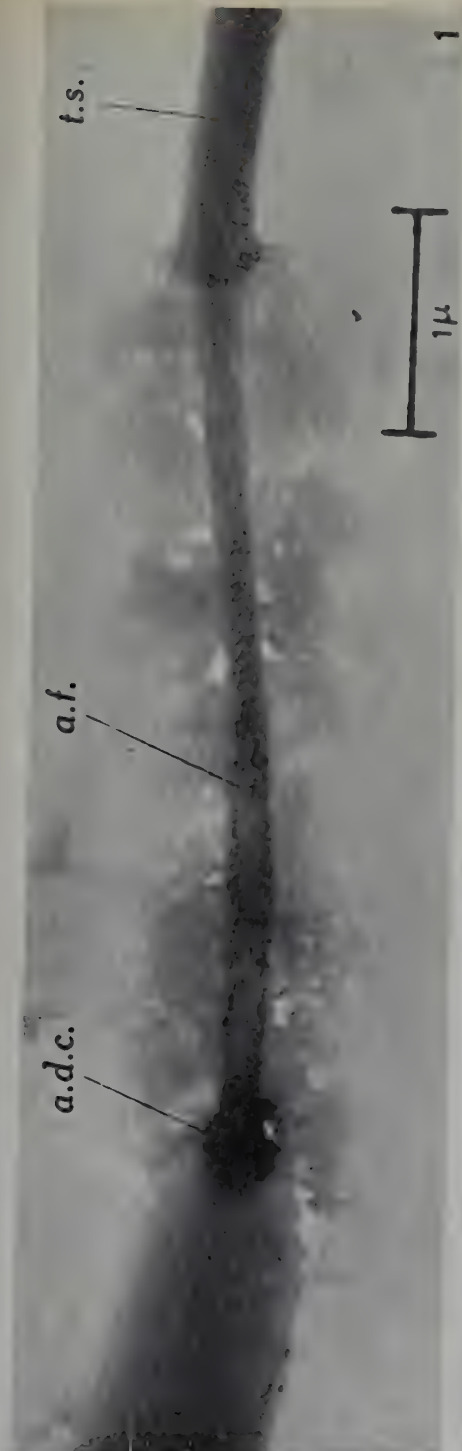


GRIGG AND HODGE.—ELECTRON MICROSCOPIC STUDIES OF SPERMATOOZOA

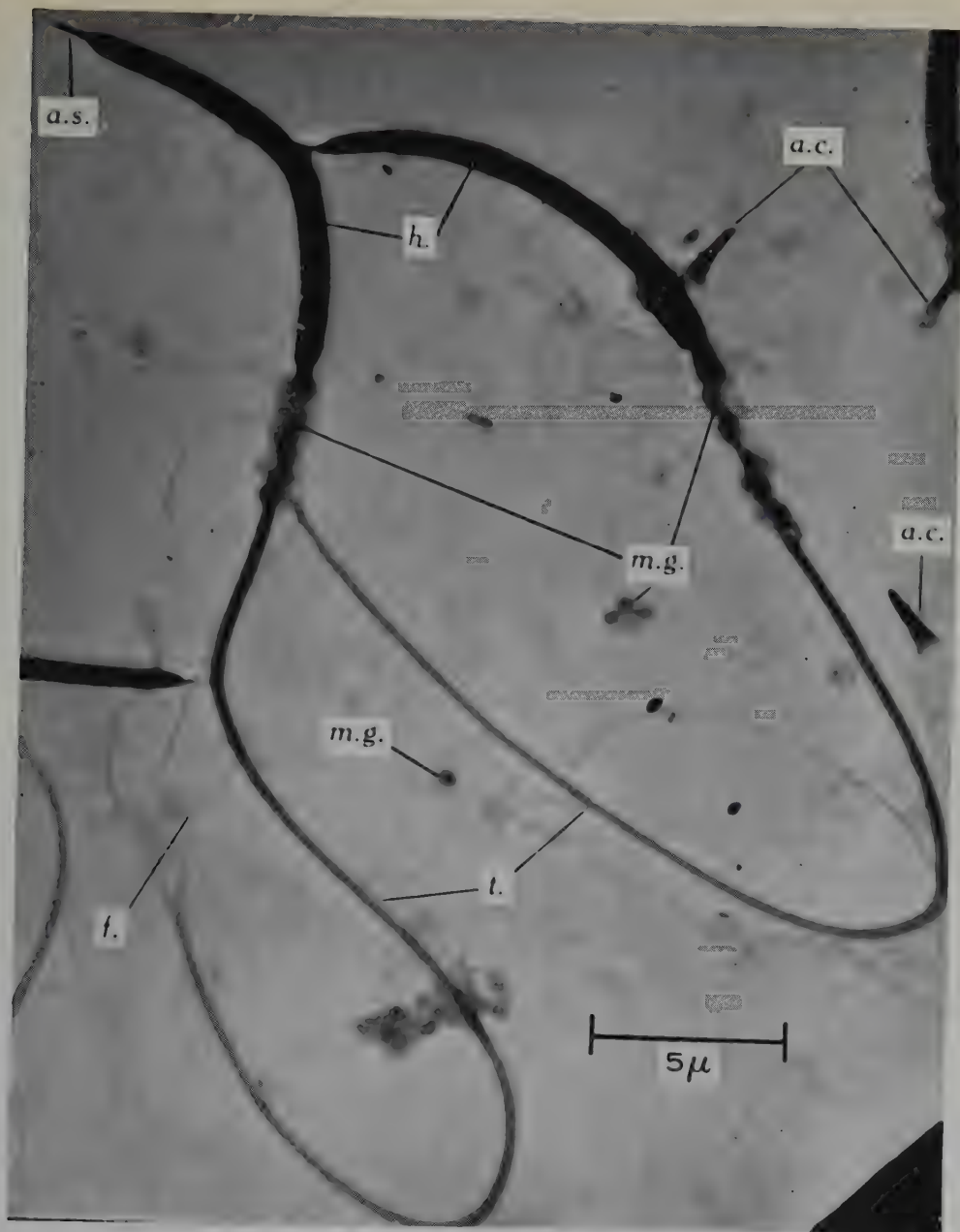




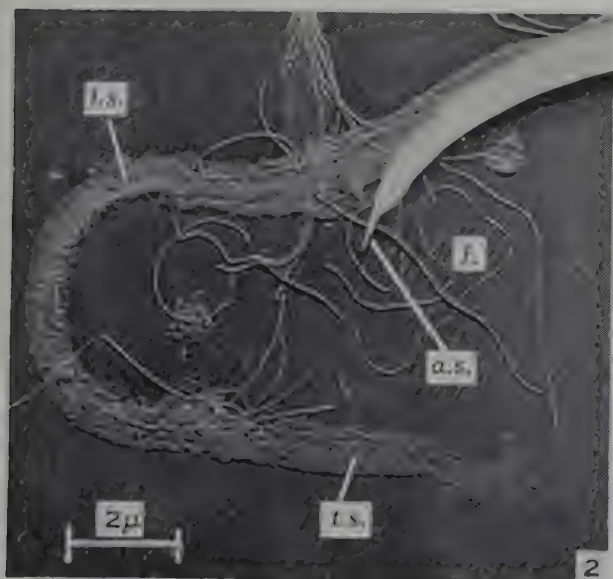
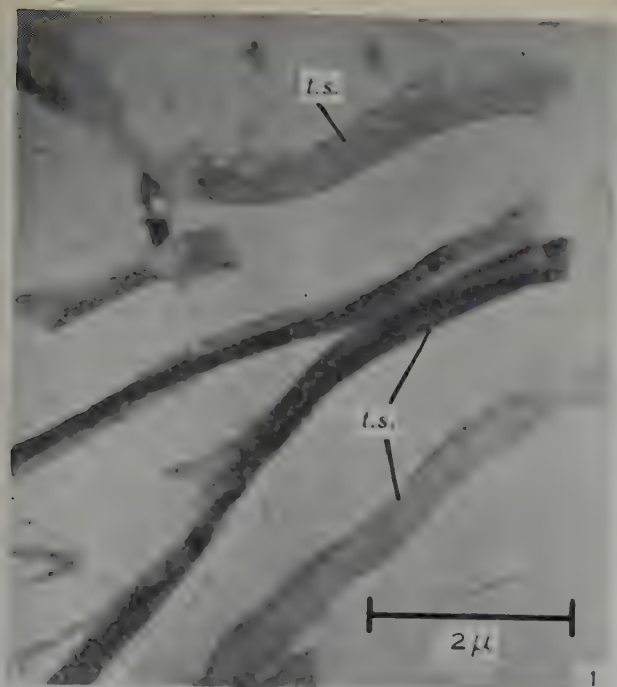
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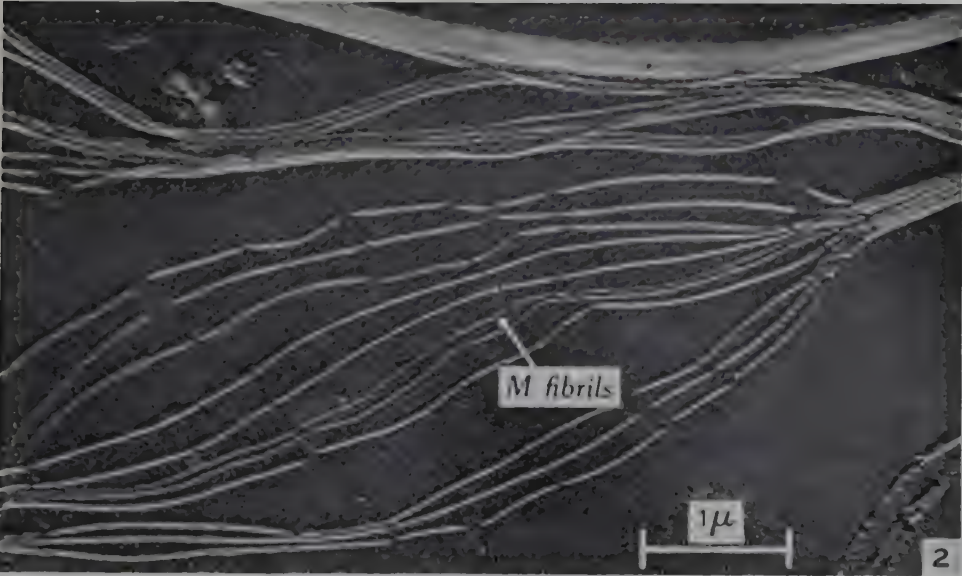
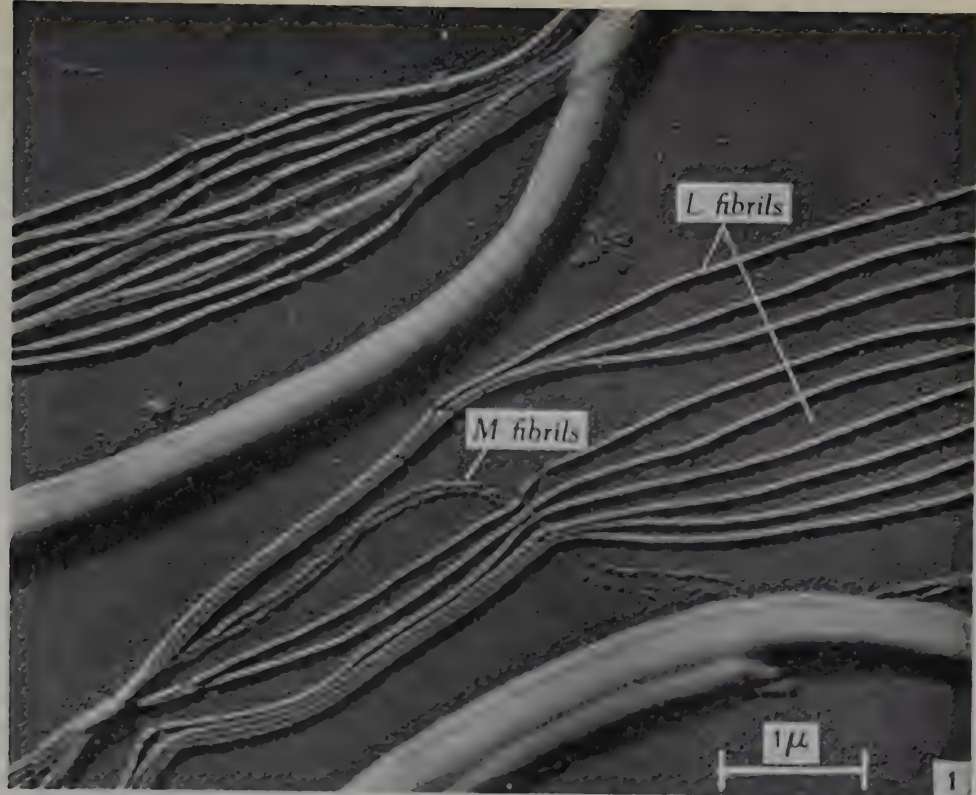
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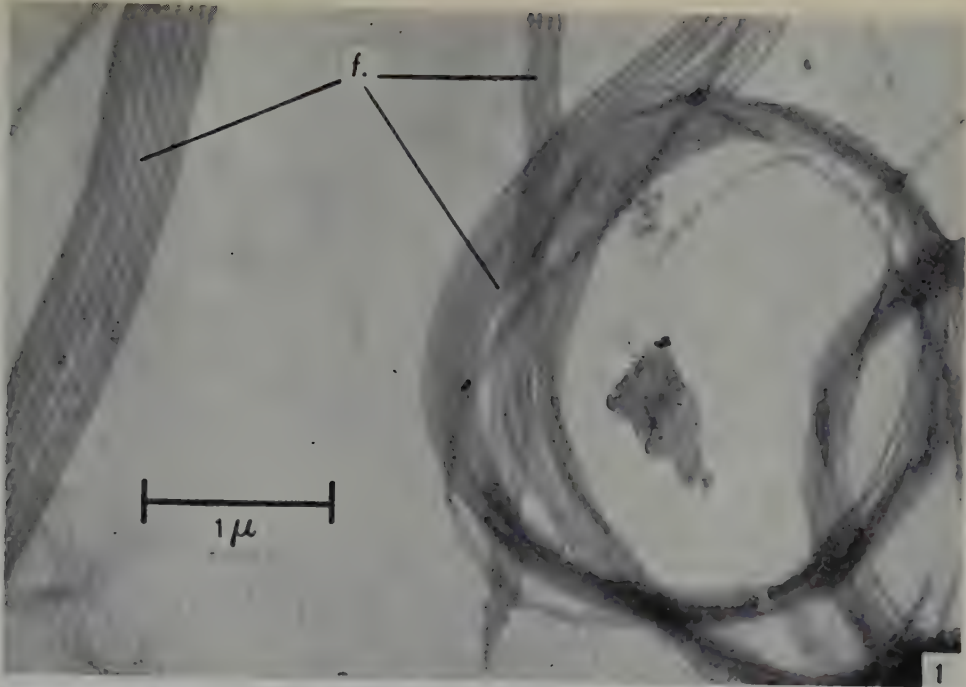
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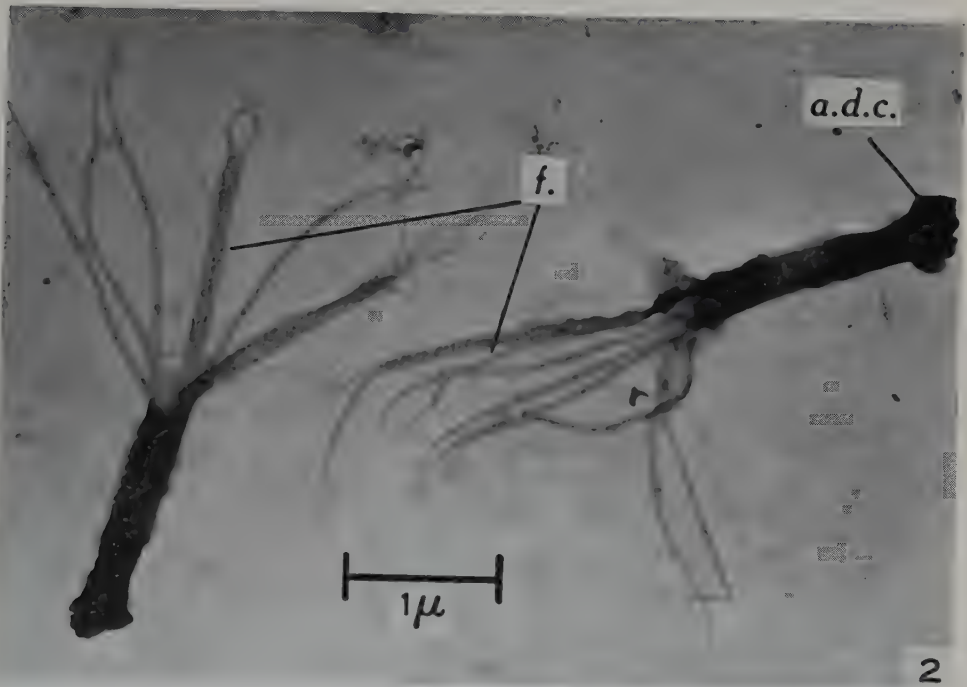
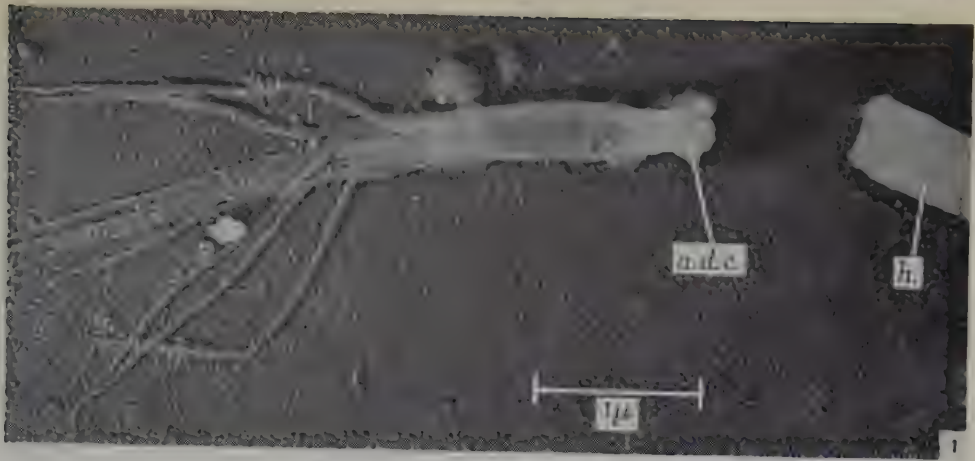
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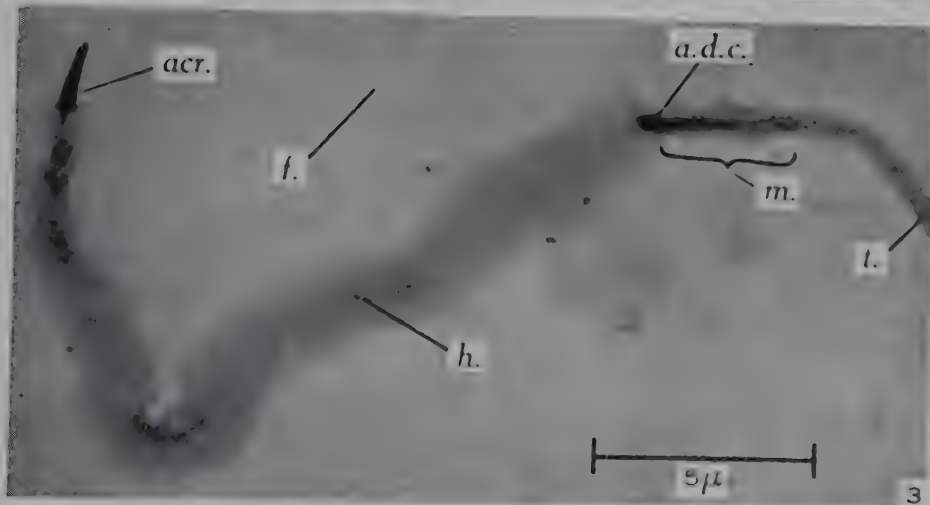
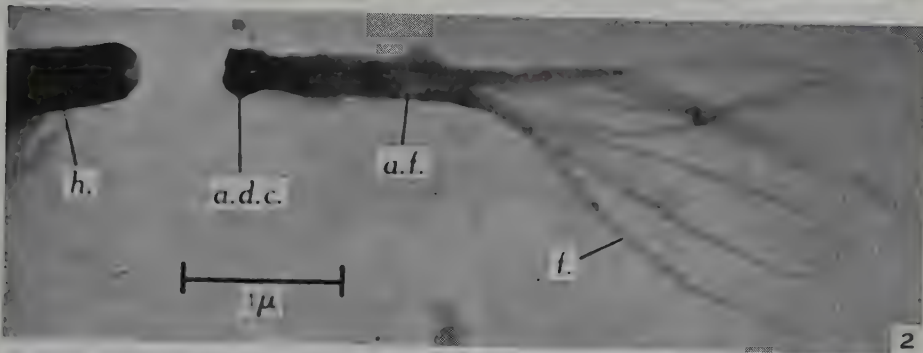
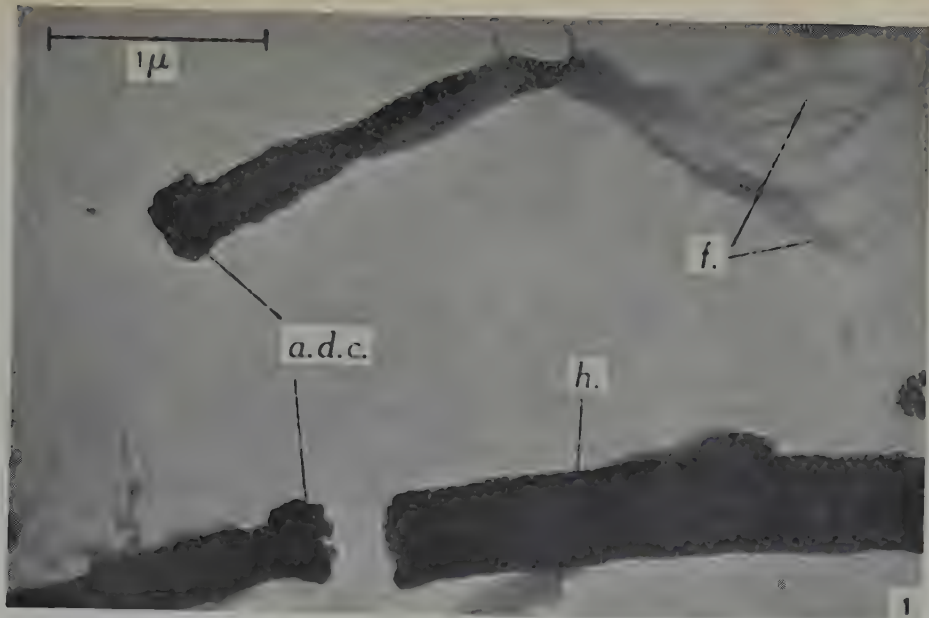
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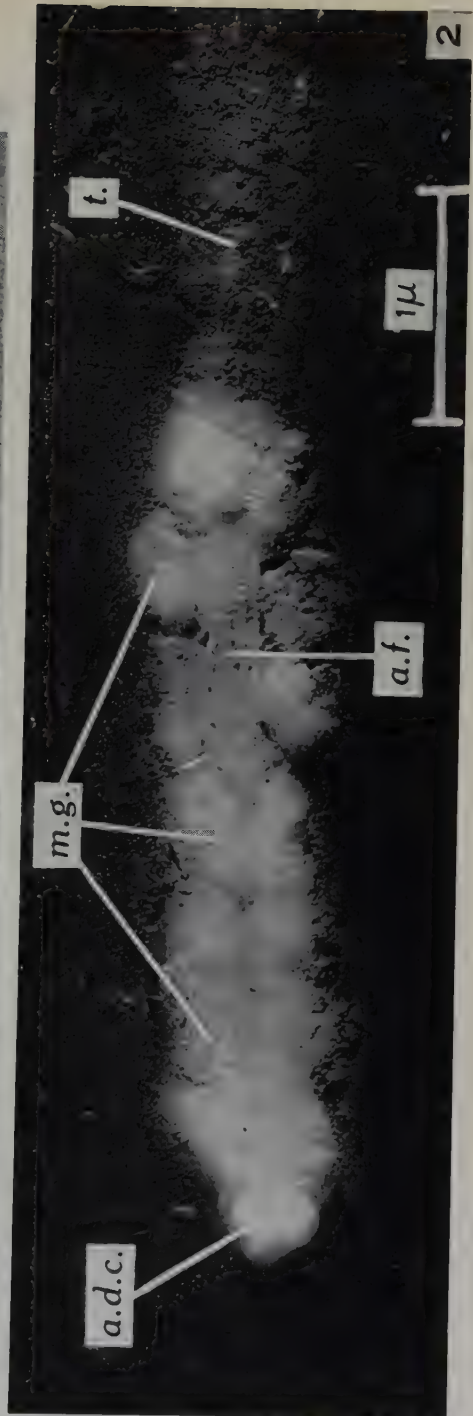
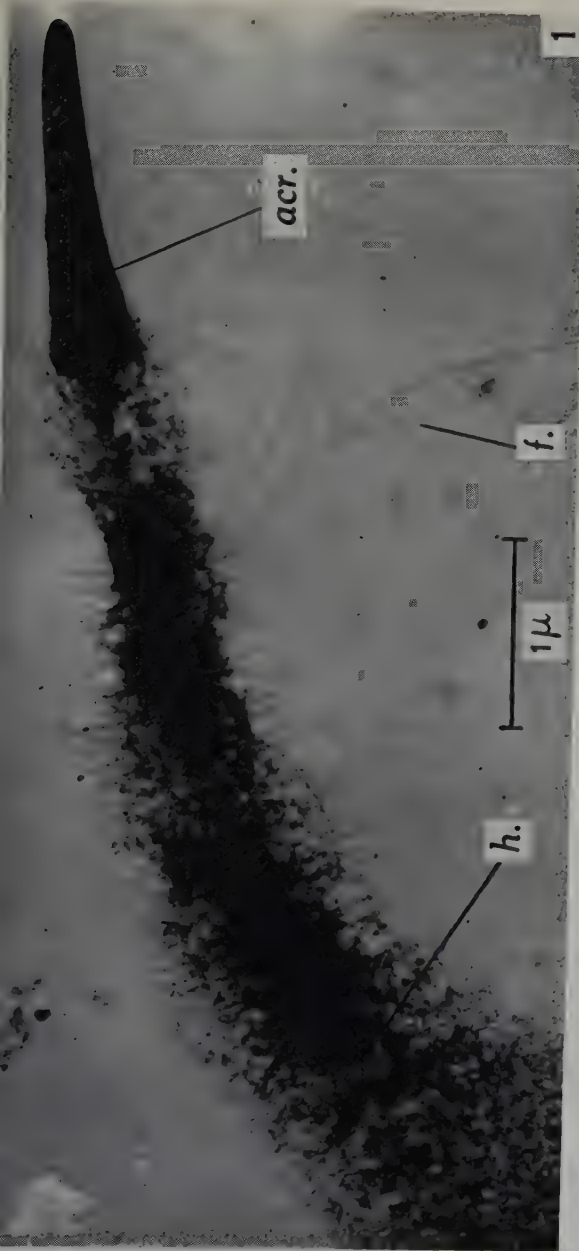


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GRIGG AND HODGE.—ELECTRON MICROSCOPIC STUDIES OF SPERMATOOZOA





GRIGG AND HODGE.—ELECTRON MICROSCOPIC STUDIES OF SPERMATOOZA

THE BIOLOGICAL SIGNIFICANCE OF HAEMOGLOBIN IN NEMATODE PARASITES

I. THE CHARACTERISTICS OF THE PURIFIED PIGMENTS

By W. P. ROGERS*

[Manuscript received March 30, 1949]

Summary

Haemoglobins from *Nippostrongylus muris*, *Nematodirus* spp., and *Haemonchus contortus* were purified by ammonium sulphate fractionation and their properties examined. All the haemoglobins showed a very high affinity for oxygen; the tension of half saturation (p_{50}) for *Nematodirus* haemoglobin of concentrations about 1×10^{-4} g.-atoms of iron per l. at pH 7.4 was in the region of 0.04 mm. of mercury. The p_{50} for *H. contortus* haemoglobin was similar to that of *Nematodirus* spp.; *N. muris* haemoglobin had a somewhat higher p_{50} . The parasite haemoglobins all showed an unusually low affinity for carbon monoxide, the equilibrium constant, $K = [\text{HbCO}] \times p\text{O}_2 / [\text{HbO}_2] \times p\text{CO}$, having a value of about 1. The "span," the distance between the α -bands of oxyhaemoglobin and carboxyhaemoglobin, varied from 60 to 65 Å. for the three parasites. None of the haemoglobins obtained from the parasites showed properties supporting the view that there is a linear relationship between $\log K$ and the "span."

The parasite haemoglobins and their derivatives differed only very slightly from those of their host in their spectroscopic properties. However, the parasite haemoglobins all showed a very much higher affinity for oxygen and a lower affinity for carbon monoxide than the pigments prepared from host blood.

The possible physiological function of haemoglobin in the parasites is discussed.

I. INTRODUCTION

Haemoglobin has a wide but irregular occurrence throughout the animal kingdom. It is even found in the nodules of certain plants (Keilin and Wang 1945). Among nematode parasites its occurrence is not uncommon. Aducco (1889) found a haemoglobin-like pigment in *Diectophyme renale*; and *Ascaris lumbricoides* (Keilin 1925), *Trichinella spiralis* larvae (Stannard, McCoy, and Latchford 1938), *Eustrongyloides* larvae (von Brand 1937), *Strongylus* spp. (Davenport 1945), *Camallanus trispinosus*, *Falcaustra affine*, and *Cruzia testudinus* (Wharton 1941) all contain haemoglobins which, however, have not been fully examined except those from *Ascaris lumbricoides* (Davenport 1945).

It has been suggested (Davey 1938; Wharton 1941) that the haemoglobins in nematode parasites may act as effective oxygen carriers. This is a most important suggestion, for it is clearly related to the question as to how small nematode parasites of the alimentary tract obtain oxygen for aerobic processes.

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Rogers (1949) has indicated that *Nippostrongylus muris* can utilize oxygen at the partial pressures which occur in the host's small intestinal fluids. It was also suggested that *Nematodirus* spp. and, to a less extent, *Haemonchus contortus* may be able to use aerobic mechanisms *in vivo*, though to a smaller degree than the rat parasite. The present work is concerned with the examination of the haemoglobins from *Nippostrongylus muris*, *Nematodirus* spp., and *Haemonchus contortus* to determine, if possible, the importance of the physiological roles of the pigments. In the present paper the purification and the properties of haemoglobin from the three parasites are described. A later paper will give the results obtained in an examination of the behaviour of the haemoglobins in the intact parasites and of their efficiency as oxygen carriers.

II. METHODS

The biological materials were obtained by the procedures described previously (Rogers 1949).

Haemoglobin was estimated as pyridine haemochromogen using a wedge trough and comparison spectroscope (Elliot and Keilin 1934). The determinations were carried out in a solution containing a final concentration of 0.01N sodium hydroxide, 20 per cent. pyridine, and a little sodium dithionite.

Absorption spectra were obtained using a General Electric recording spectrophotometer over the range 400-700 m μ . with either the per cent. transmission, density, or log₁₀ density cams.

The special procedure used in obtaining the oxygen dissociation curves of the haemoglobins and the equilibrium constants $K = [\text{HbCO}] \times \text{pO}_2 / [\text{HbO}_2] \times \text{pCO}$ will be described later in this paper.

(a) The Extraction and Purification of Haemoglobin

Washed parasites were ground in a mortar with a little water, sodium dithionite, and powdered glass.* The ground tissue was then extracted twice with water, the debris being removed by centrifuging. The fatty layer which was formed at the surface of the haemoglobin solution when it was centrifuged was removed and the pH adjusted to 6.8. Solid ammonium sulphate was added to the opalescent solution of the haemoglobin to 70 per cent. saturation and the pH again brought to 6.8. The solution was then chilled on ice until the precipitate aggregated and could be removed by centrifuging. More solid ammonium sulphate was added to 90 per cent. saturation, the precipitated haemoglobin was collected by centrifuging, dissolved in a little water, and dialysed against distilled water adjusted to pH 7.4 at 5°C. until free from sulphate. Any precipitate formed during dialysis was removed by centrifuging.

The solutions so obtained contained oxyhaemoglobin with small and variable amounts of methaemoglobin and choleglobin. These pigments could be further purified by precipitation with ammonium sulphate between 75 and 85 per cent.

* It was found that the addition of sodium dithionite in small amounts did not cause an appreciable increase in the choleglobin content of the final preparation and the reducing agent was therefore added as a routine measure to minimize oxidation while the tissue was being ground.

saturation. This procedure, however, gave preparations which contained large amounts of methaemoglobin and was not suitable for most of the experiments. As a rule the haemoglobin was prepared without the second precipitation with ammonium sulphate.

Of the parasite haemoglobins examined it was found that *Nippostrongylus muris* was most resistant to oxidation during preparation, whereas *Haemonchus contortus* haemoglobin was by far the most sensitive. *Nematodirus* haemoglobin was most easily obtained in large amounts and was more thoroughly examined than that from the other parasites.

Attempts to obtain crystalline methaemoglobin by several different methods all failed.

III. PROCEDURE AND RESULTS

(a) The Absorption Characteristics of the Parasite Haemoglobins

The absorption curves were obtained with a recording spectrophotometer in the region 400 to 700 m μ . using a log₁₀ density cam. Before use, the pigment

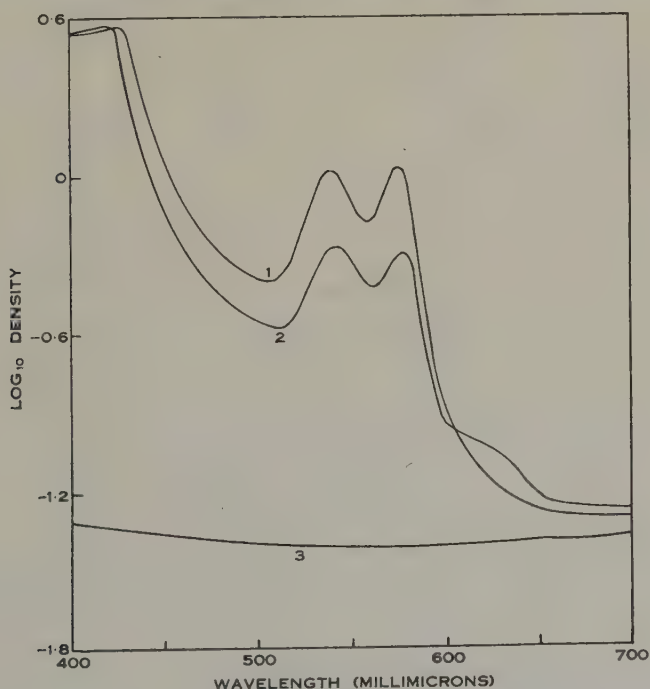


Fig. 1.—Log₁₀ density curves of sheep blood oxyhaemoglobin (1), *Nematodirus* spp. oxyhaemoglobin (2), and water (3) in a 1 cm. cell. Small amounts of methaemoglobin and choleglobin were present in the parasite preparation.

solution was adjusted to pH 8.0 with sodium hydroxide and buffered with M/100 phosphate of the same pH. Solutions were examined in a 1 cm. cell.

Wherever possible, enough solution was prepared to allow all the haemoglobin derivatives to be examined at the one concentration. In each case, the absorption spectrum was compared with that taken from the pigment prepared from host blood by ammonium sulphate precipitation. The form of the curves taken with the \log_{10} density cam was not affected by the concentration of the pigments and it was therefore convenient to use concentrations which allowed the curves of host and parasite haemoglobin derivatives to be shown distinctly in each figure. The curves shown in Figures 1, 2, 3, and 4 show (1) host haemoglobin derivative, (2) parasite haemoglobin derivative, and (3) the medium used (M/100 phosphate buffer).

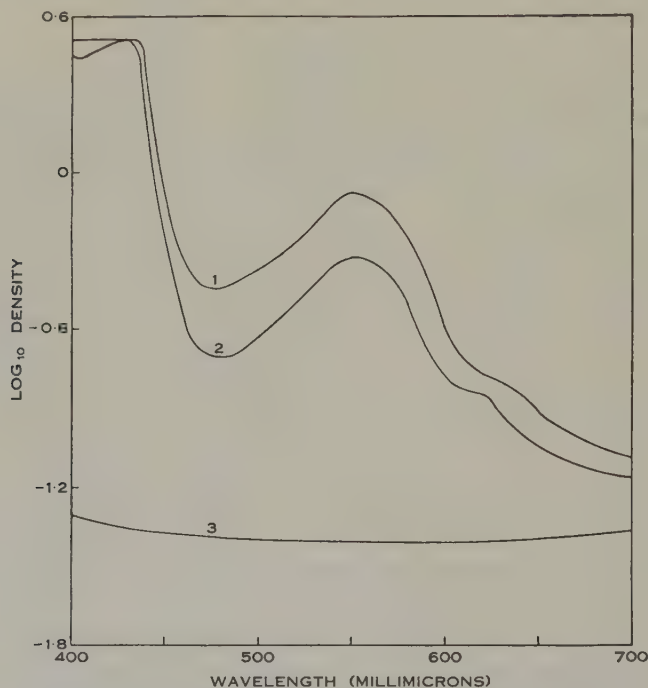


Fig. 2.— Log_{10} density curves of sheep blood haemoglobin (1), *Nematodirus* spp. haemoglobin (2), and water (3) in a 1 cm. cell. A small amount of choleglobin was present in the parasite preparation.

(i) *Oxyhaemoglobin*.—The absorption spectra of sheep and *Nematodirus* spp. oxyhaemoglobin are shown in Figure 1. The parasite oxyhaemoglobin contained substances which were not present in the preparation from host blood and which caused the difference in the curves at the longer wavelengths. The contaminating substances were alkaline methaemoglobin and choleglobin, the band at 620 $\text{m}\mu$ probably being produced by choleglobin.

(ii) *Haemoglobin*.—This derivative was prepared by adding a little sodium dithionite buffered at pH 8.0. The only interfering substance in the parasite

preparation was probably choleglobin, which again gave the band at 620 m μ (see Fig. 2).

(iii) *Carboxyhaemoglobin*.—The absorption curves are shown in Figure 3. Carbon monoxide was passed through the haemoglobin solution and a little sodium dithionite added. Choleglobin was again the chief contaminant in the parasite haemoglobin.

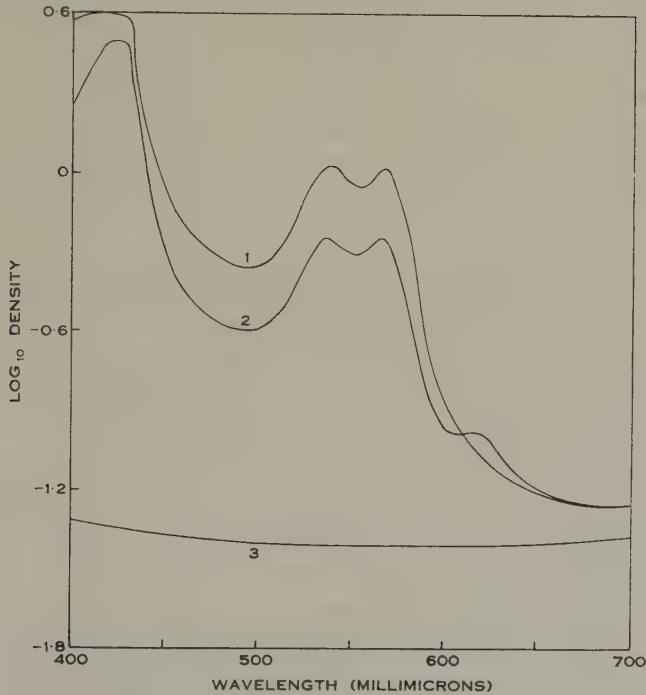


Fig. 3.—Log₁₀ density curves of sheep blood carboxyhaemoglobin (1), *Nematodirus* spp. carboxyhaemoglobin (2), and water (3) in a 1 cm. cell. A small amount of choleglobin was present in the parasite preparation.

(iv) *Alkaline Methaemoglobin*.—This derivative, which was prepared by adding a little sodium ferricyanide at pH 8.5, gave the absorption spectra shown in Figure 4. Similar curves were obtained at pH 9.5. Borate buffers were used.

The wavelengths of the chief bands of oxyhaemoglobin, carboxyhaemoglobin, and haemoglobin from *Haemonchus contortus*, *Nematodirus* spp., and *Nippostrongylus muris* are shown in Table 1. Spectroscopically the haemoglobin derivatives of the parasites were all very similar to those of the host animals but they did differ in certain minor characteristics. Also there were slight differences between absorption spectra of the haemoglobin derivatives of the three parasites. Spectra of the reduced pyridine haemochromogens prepared from the parasites, and from the hosts' blood were identical. It would appear that it was only the protein moiety of the haemoglobins which differed.

(b) *The "Span" of the Parasite Haemoglobins*

The distance between the α -bands of the parasite oxyhaemoglobin and carboxyhaemoglobin, the "span" (Anson *et al.* 1924), is shown in Table 2. The

TABLE 1
THE POSITION OF THE CHIEF ABSORPTION BANDS OF THE DERIVATIVES OF HAEMOGLOBIN FROM NIPPOSTRONGYLUS MURIS, NEMATODIRUS SPP., AND HAEMONCHUS CONTORTUS.
WAVELENGTHS ARE GIVEN IN MILLIMICRONS

Species of Parasite	Oxyhaemoglobin		Carboxyhaemoglobin		Haemoglobin
	α -band	β -band	α -band	β -band	
<i>Nippostrongylus muris</i>	575	541	569	538.5	556
<i>Nematodirus</i> spp.	576.5	542	570	537	555
<i>Haemonchus contortus</i>	576	541	570	538	554

TABLE 2
THE "SPAN," THE DISTANCE IN ANGSTROM UNITS BETWEEN THE α -BANDS OF OXYHAEMOGLOBIN AND CARBOXYHAEMOGLOBIN PREPARED FROM A NUMBER OF DIFFERENT ORGANISMS

Origin of Haemoglobin	Span (\AA)	Observers
Blood of vertebrates	43-56	Anson <i>et al.</i> (1924)
Muscle of vertebrates	31-36	Roche (1933), Theorell (1934 <i>a</i> , 1934 <i>b</i>)
Root nodules of leguminous plants	100	Keilin and Wang (1945)
<i>Gastrophilus intestinalis</i> larvae	95	Keilin and Wang (1946)
<i>Nippostrongylus muris</i>	60	Rogers (present paper)
<i>Nematodirus</i> spp.	65	"
<i>Haemonchus contortus</i>	64	"
<i>Arenicola</i>	54	Barcroft (1928)
<i>Lumbricus</i>	41	"
<i>Planorbis</i>	44	"
<i>Chironomus</i>	56	"

results given are averages of 10 determinations made with a Hartridge spectrometer using two samples of pigment from each of the parasites which were examined.

(c) *The Determination of Oxygen Dissociation Curves of the Parasite Haemoglobins*

The dissociation of oxyhaemoglobin at different partial pressures of oxygen was determined spectrophotometrically using the cell shown in Figure 5A. The

total volume of the cell was determined by weighing with and without mercury, and the neck of the cell was calibrated so that the volume of haemoglobin solution placed in the cell could be measured. The haemoglobin was buffered

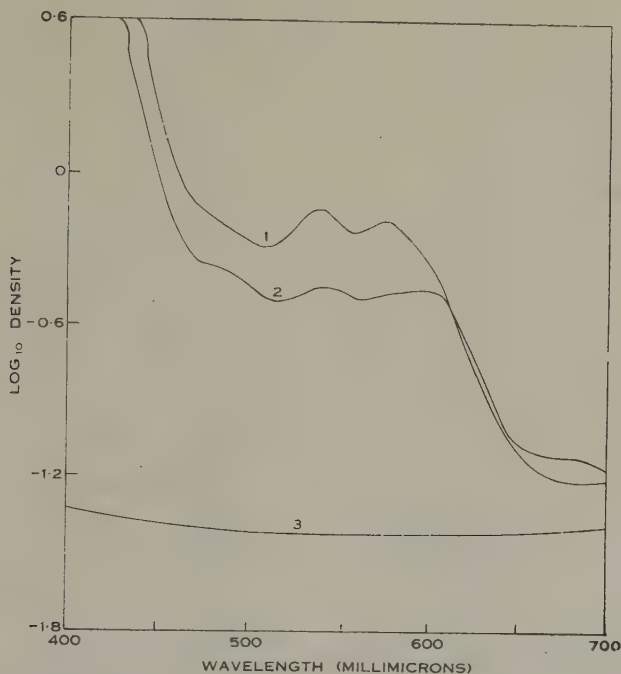


Fig. 4.— Log_{10} density curves of sheep blood alkaline methaemoglobin (1), *Nematodirus* spp. alkaline methaemoglobin (2), and water (3) in a 1 cm. cell. A small amount of choleglobin was present in the parasite preparation. The solutions were buffered at pH 8.5.

with M/100 phosphate at pH 7.4 and was examined at a temperature of 16–17°C. After placing the haemoglobin in the cell the gas pressure was reduced to about 5 mm. of mercury, and nitrogen, which had been freed of oxygen by passage over copper filings at 400°C., let into the cell. This procedure was repeated three times, after which the pressure of the nitrogen in the cell was reduced to about 3 mm. of mercury. The solution in the cell was then equilibrated with the gas phase by gentle rocking in an incubator at 37°C. for about 30 minutes. The whole procedure of evacuation, filling with nitrogen, evacuation, and equilibration at 37°C. was repeated three times, after which the cell was filled with oxygen-free nitrogen at a slight negative pressure. Such a procedure led to the deoxygenation of 91 to 98 per cent. of the oxyhaemoglobin. Unfortunately, methaemoglobin formation during deoxygenation was very marked, especially with haemoglobin from *Haemonchus contortus*, when the methaemoglobin sometimes formed 60 per cent. of the total haemoglobin present.

Using the density cam on the recording spectrophotometer, the absorption spectra of the haemoglobin solution were obtained before and after adding small amounts of oxygen in distilled water from a pipette to the contents of

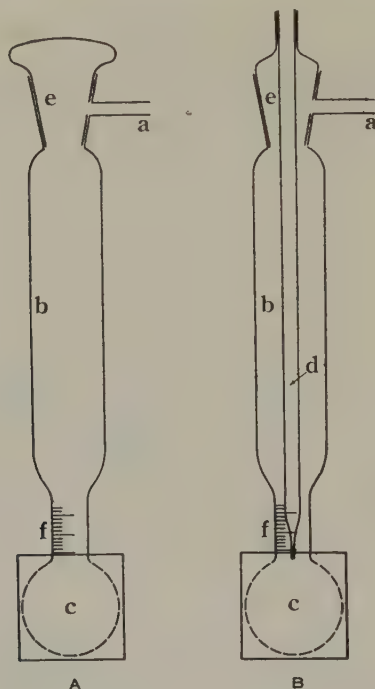


Fig. 5.—The tubes, with cells attached, used in the determination of A, the oxygen dissociation curves of the parasite haemoglobins, and B, the partition constant $K = [\text{HbCO}] \times \text{pO}_2 / [\text{HbO}_2] \times \text{pCO}$; a, the side arms of the tubes closed by turning the stoppers, e; b, the tubes graduated at the neck, f, and attached to cells, c, 1 cm. wide; d, inlet for passing carbon monoxide-oxygen mixtures through cell B.

the cell. A short length of rubber tubing was attached to the side arm of the cell (a) (see Fig. 5) and was completely filled with distilled water of known oxygen content, determined by the Winkler method (Krogh 1935). A graduated 1 ml. pipette was then forced into the rubber tubing causing the water to rise in the pipette. As the gas in the cell was at a slight negative pressure a small amount of water could be accurately let into the cell. After removing the water left in the side arm of the cell, the haemoglobin was equilibrated with the gas phase and the absorption spectra taken. This procedure was repeated several times until finally the cell was opened and equilibrated with air and the curve

recorded. The absorption curves were also taken after the addition of ferricyanide and dithionite. In Figure 6 a set of absorption curves is shown. The conditions under which each curve was obtained are shown in Table 3.

TABLE 3
CONDITIONS UNDER WHICH THE ABSORPTION CURVES OF FIGURE 6 WERE OBTAINED

Curve No.*	Conditions for obtaining the Curve	Chief Components of the Solution
1	Gaseous oxygen removed	Haemoglobin and methaemoglobin
2, 3, 4, 5	By the successive addition of 0.1 ml. lots of water containing 17.6×10^{-4} ml. oxygen	Oxyhaemoglobin, haemoglobin, and methaemoglobin
6	After equilibration with air	Oxyhaemoglobin and methaemoglobin
7	By adding potassium ferricyanide	Methaemoglobin
8	By adding sodium dithionite	Haemoglobin
9	Solution in the cell replaced by water	Water only
10	Cell removed	Per cent. transmission curve of spectrophotometer

* Curves 1-9 were obtained with the density cam, curve 10 with the per cent. transmission cam.

The amounts of water added to the cell were minimized by using water with a high oxygen content. It was found that water through which gaseous oxygen had been passed for about five minutes retained a constant oxygen concentration over a period of two hours if it was stored in completely filled glass-stoppered Erlenmeyer flasks. Water from a freshly opened flask was used for each addition to the cell.

The ratios of the concentrations of oxyhaemoglobin to the haemoglobin in curves 1 to 5 were calculated as follows: Let C_1 , C_2 , and C_3 be the concentrations of oxyhaemoglobin, methaemoglobin, and haemoglobin respectively, of specific extinction coefficients $\epsilon_{x_1}^x$, $\epsilon_{x_2}^x$, and $\epsilon_{x_3}^x$ at a wavelength λ_x m μ . Let $d_{x_b}^x$ be the observed density of a haemoglobin solution, $d_{x_a}^x$ that at concentration C_a , and $d_{x_w}^x$ the density of the cell containing the water medium alone (curve 9) at λ_x . Then, if the addition of water to the contents of the cell changed the haemoglobin concentration from $C_a = C_1 + C_2 + C_3$ to C_b , true isosbestic points could be found by trial and error from the formula

$$d_{x_a}^x = \frac{C_a}{C_b(d_{x_b}^x - d_{x_w}^x)} \cdot$$

By this means, the true isosbestic points for curves 1 to 6 of Figure 6 were found to be at λ_{504} , λ_{550} and λ_{570} . At such points $\epsilon^x_1 = \epsilon^x_3$ because oxyhaemoglobin and haemoglobin were the only components which were changed in concentration. By means of the formula

$$d^x_a = \epsilon^x_3 C_3$$

ϵ^x_3 could be found from curve 8 (haemoglobin). Hence ϵ^x_1 could be found at λ_{504} , λ_{550} , and λ_{570} . Further, ϵ_2 could be found for any wavelength from curve 7 obtained from methaemoglobin. Thus the formula

$$d^x_a = \epsilon^x_1 C_1 + \epsilon^x_2 C_2$$

at λ_{504} , λ_{550} , and λ_{570} could be applied to curve 6 obtained from a mixture of oxyhaemoglobin and methaemoglobin, and the proportions of C_1 and C_2 found.

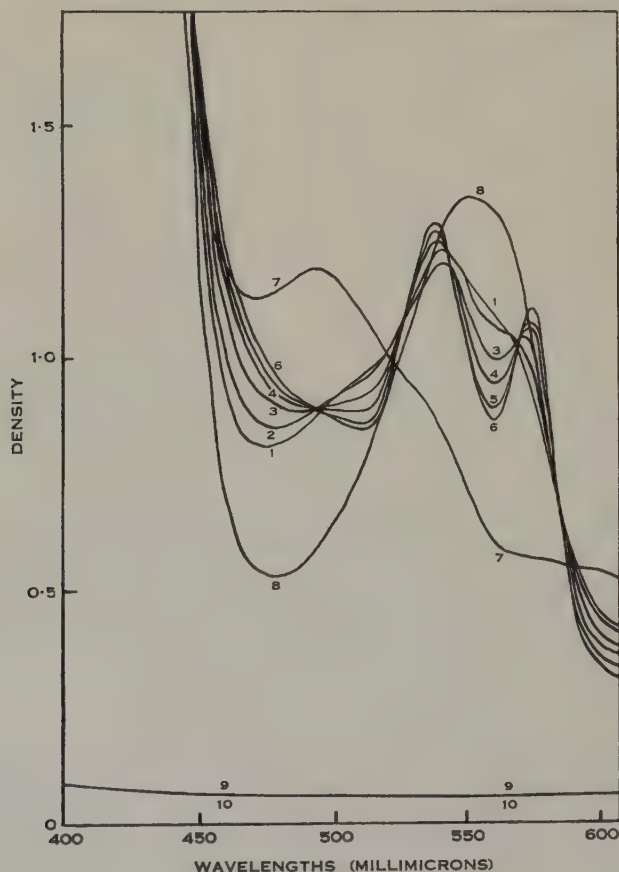


Fig. 6.—A series of curves, 1-9, taken with the density cam for the determination of the oxygen dissociation curve of *Nematodirus* spp. haemoglobin. Curve 10 shows the 100 per cent. transmission characteristic of the spectrophotometer and almost exactly follows the base line. The conditions under which each curve was obtained and the nature of the haemoglobin derivatives formed are listed in Table 3.

For further information see text.

The results obtained were: C_1/C_2 at $\lambda_{504} = 0.55/0.45$; at $\lambda_{550} = 0.56/0.46$; and at $\lambda_{570} = 0.56/0.45$. Since the proportion of methaemoglobin did not change in curves 1-6, the ratio of $(C_1 + C_3)/C_2$ could be taken as 0.55/0.45 for them all. Suitable points, λ_{550} and λ_{563} , were then chosen for determining the proportions of oxyhaemoglobin and haemoglobin giving curves 1-5. ϵ_1^{563} was then calculated from data obtained from curve 6 using the formula

$$d_a^{563} = \epsilon_1^{563}C_1 + \epsilon_2^{563}C_2.$$

Curves 1-5 were then corrected for dilution at λ_{563} caused by the addition of distilled water. Then, applying the formula

$$d^x = \epsilon^x_1C_1 + \epsilon^x_2C_2 + \epsilon^x_3C_3$$

at λ_{550} and λ_{563} it was possible to calculate the proportions of C_1 and C_3 . The results obtained from Figure 6 are shown in Table 4. Knowing the total haematin present in the haemoglobin solution, which had been determined as the pyridine haemochromogen, the amounts of oxygen used to form oxyhaemoglobin on each addition of oxygen in distilled water could be calculated and the remaining oxygen, which was partitioned between the gas phase and the solution, determined. It then remained to calculate the partial pressure of oxygen at which the curves 1-5 were obtained.

Essentially, it was necessary to calculate the partial pressure of oxygen in the cell which was initially free of gaseous oxygen and into which known amounts of oxygen were introduced in distilled water. The cell contained a solution of haemoglobin which combined with known amounts of oxygen. Let V_1 ml. be the volume of the cell and V_2 ml. the volume of the haemoglobin solution. Into this introduce V_3 ml. of water containing y g. of oxygen. Let z g. of oxygen combine with the haemoglobin, and suppose x g. of oxygen enter the gas phase. Then the concentration (wt./vol.) of oxygen in the liquid phase will be $y - (x + z)/(V_2 + V_3)$ g. per ml., and the concentration of oxygen in the gas phase will be $x/V_1 - (V_2 + V_3)$. But

$$\frac{[\text{O}_2] \text{ in g. per ml. liquid phase}}{[\text{O}_2] \text{ in g. per ml. gas phase}} = \text{a constant} = 0.03348$$

at 16°C. and 760 mm. pressure at which the reactions were carried out, assuming that the solubility of oxygen in the water of a dilute haemoglobin solution is similar to its solubility in pure water. Hence

$$\frac{y - (z + x)}{V_2 + V_3} \bigg/ \frac{x}{V_1 - (V_2 + V_3)} = 0.03348.$$

The value of x could thus be calculated and the partial pressure of oxygen in the gas phase of volume $V_1 - (V_2 + V_3)$ ml. obtained.

The results obtained with the two samples of haemoglobin from *Nematodirus* spp. are shown in Figure 7. The oxygen tension at which the haemoglobin of *Nematodirus* spp. and *Haemonchus contortus* was 50 per cent. saturated was in the region of 0.05 mm. of mercury; for *Nippostrongylus muris* the figure was 3 to 4 times that of the sheep parasites. The haemoglobin solutions used had a concentration in the region of 1×10^{-4} g.-atoms of iron per litre.

The method described above had certain advantages over that described by Hill (1936) in that the observations were made spectrophotometrically, and, though more elaborate calculations were necessary, it was possible to determine

TABLE 4
THE RELATIVE AMOUNTS OF OXYHAEMOGLOBIN, HAEMOGLOBIN, AND
METHAEMOGLOBIN OBTAINED FROM THE CURVES OF FIGURE 6

Curve No.	Oxyhaemoglobin	Haemoglobin	Methaemoglobin
1	0.05	0.53	0.45
2	0.07	0.50	0.45
3	0.28	0.28	0.45
4	0.42	0.15	0.45
5	0.51	0.05	0.45
6	0.55	None	0.45

the dissociation curves in the presence of methaemoglobin. The addition of oxygen in distilled water, instead of oxyhaemoglobin, to the cell was necessary because the long periods of equilibration and evacuation caused considerable changes in the methaemoglobin concentration. However, the results given above must be considered as approximate only, because the presence of choleglobin, especially in the preparations from *Nematodirus* spp. and *Haemonchus contortus*, probably caused considerable error. Further, the haemoglobin used to obtain curve 1 was never more than 98 per cent. reduced and the zero point had to be obtained by extrapolation.

(d) *The Relative Affinity of Parasite Haemoglobin for Oxygen and Carbon Monoxide*

From the reversible equation



the equilibrium constant

$$K = [\text{HbCO}] \times p\text{O}_2 / [\text{HbO}_2] \times p\text{CO} \quad \dots \quad (1)$$

was found by the following method:

A solution of partially purified pigment of suitable concentration in M/100 phosphate buffer at pH 7.4 was placed in a special cell (See Fig. 5B). The absorption spectrum, which was predominantly that of oxyhaemoglobin with small amounts of methaemoglobin and choleglobin, was then taken using the density cam. A known mixture of oxygen and carbon monoxide was passed through the oxyhaemoglobin solution, the cell closed, and the solution and gas equilibrated. The absorption curve, which differed from the first in that some of the oxyhaemoglobin had been changed into carboxyhaemoglobin, was then taken. The gas phase was then replaced with pure carbon monoxide and equilibrated with the solution. The three curves obtained in one experiment

are shown in Figure 8. The curve *a* was from oxyhaemoglobin, *b* from oxyhaemoglobin and carboxyhaemoglobin, and *c* from carboxyhaemoglobin. All three solutions contained the same small amounts of methaemoglobin and choleglobin; that the changes occurring when gas was passed through the cell were largely confined to two components, the oxyhaemoglobin and the carboxyhaemoglobin, is indicated by the isosbestic points at 536, 551, 573, and 596 m μ .

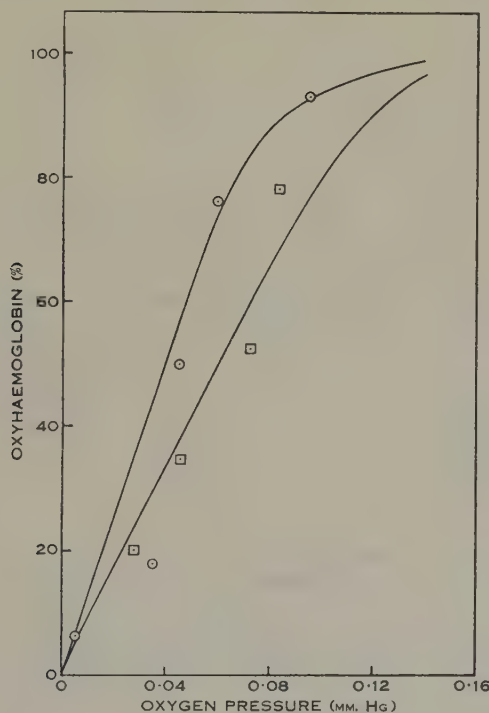


Fig. 7.—The oxygen dissociation curves of *Nematodirus* spp. haemoglobin at concentrations of 0.7×10^{-4} g.-atoms of iron per l. (circles) and 1.0×10^{-4} g.-atoms of iron per l. (squares) in M/100 phosphate buffer at pH 7.4. Owing to the presence of choleglobin in the preparations used the results cannot be considered to have a high accuracy; they are included to indicate the approximate position of the curve in relation to the partial pressure of oxygen rather than the actual shape of the curve.

The calculations to determine the changes in the oxyhaemoglobin and carboxyhaemoglobin concentrations taking place in the presence of methaemoglobin, were carried out as follows, using the same notation as given previously except that C_3 and ϵ_3 refer to carboxyhaemoglobin instead of haemoglobin.

Let $C = C_1 + C_3$, then, at λ_x , from curve a ,

$$d^x_a = \epsilon^x_1 C + \epsilon^x_2 C_2. \quad \dots \dots \dots (2)$$

Again, at λ_x , from curve b ,

$$d^x_b = \epsilon^x_1 C_1 + \epsilon^x_3 (C - C_1) + \epsilon^x_2 C_2 \quad \dots \dots (3)$$

and from curve c ,

$$d^x_c = \epsilon^x_3 C + \epsilon^x_2 C_2 \quad \dots \dots \dots (4)$$

Subtract (2) from (4) and (3) from (4) and obtain

$$d^x_c - d^x_a = C(\epsilon^x_3 - \epsilon^x_1) \quad \dots \dots \dots (5)$$

and

$$d^x_c - d^x_b = C_1(\epsilon^x_3 - \epsilon^x_1) \dots \dots \dots (6)$$

Then from (5) and (6),

$$\frac{d^x_c - d^x_a}{d^x_c - d^x_b} = \frac{C}{C_1} = \frac{[\text{HbO}_2] + [\text{HbCO}]}{[\text{HbO}_2]} \quad \dots \dots \dots (7)$$

Using equation (7), $[\text{HbO}_2]/[\text{HbCO}]$ could thus be calculated from the curves given in Figure 8, and K could be obtained from equation (1) because $p\text{O}_2$ and $p\text{CO}$ were known.

Using λ_{560} and λ_{540} the values of K from two experiments were found to be 1.4 and 2.0, 0.5 and 0.6 for *Nematodirus* spp. The variation in the results was probably due to interfering substances such as choleglobin. The equilibrium constant for *Haemonchus contortus* was of the same order as that of *Nematodirus* spp.

As an alternative to the method given above, the curve of carboxyhaemoglobin uncontaminated with methaemoglobin was obtained by adding sodium dithionite after passing pure carbon monoxide through the cell. The absorption curve of methaemoglobin was also taken. It was then possible to determine $\epsilon^x_1 C_1$ and ϵ^x_3 directly. Such a procedure was used only once; it gave results similar to that obtained with the first method.

IV. DISCUSSION

The haemoglobins of *Nippostrongylus muris*, *Nematodirus* spp., and *Haemonchus contortus* all had tensions of half saturation (p_{50} 's) which were of the same order as that of dilute *Gastrophilus* haemoglobin (Keilin and Wang 1946) though higher than that of *Ascaris lumbricoides* (Davenport 1945). Compared with the haemoglobins from host blood which had p_{50} 's in the region of 4 (rat) and 7 (sheep) mm. of mercury in dilute solution at pH 7.4 (Hill and Wolvekamp 1936), the parasite haemoglobins had a very high affinity for oxygen. Wharton (1941) found the p_{50} for *Camallanus trispinosus* haemoglobin to be about 8 mm. of mercury at pH 6.8. Although it is not clear what concentration of haemoglobin was used it is evident that the *Camallanus* haemoglobin had a lower affinity for oxygen than most of the nematode haemoglobins. However, that there is a wide range in the p_{50} 's of the parasite haemoglobins,

from *Camallanus* to *Nippostrongylus*, *Nematodirus*, *Haemonchus*, and finally *Ascaris*, is evident.

The parasite haemoglobins were remarkable for the ease with which they were oxidized to methaemoglobin, particularly in *Nematodirus* spp. and *Haemonchus contortus*. As with vertebrate haemoglobins (Neill and Hastings

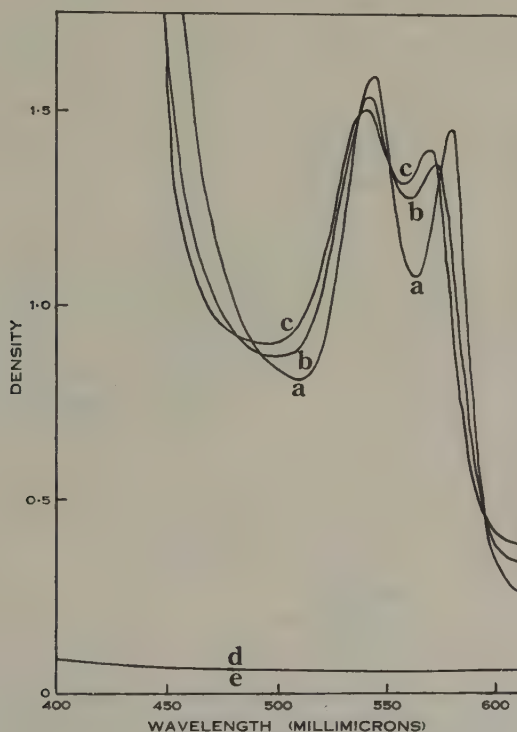


Fig. 8.—The density curves of *Nematodirus* spp. haemoglobin taken for the determination of the equilibrium constant $K = [\text{HbCO}] \times p\text{O}_2 / [\text{HbO}_2] \times p\text{CO}$ at pH 7.4; a, the curve of oxyhaemoglobin; b, the curve of mixed oxyhaemoglobin and carboxyhaemoglobin formed in the presence of a mixture of carbon monoxide and oxygen of known composition; c, the curve of carboxyhaemoglobin; d, the curve obtained with water only in the cell; e, the 100 per cent. transmission characteristic of the spectrophotometer which almost exactly follows the base line. All the haemoglobin solutions contained a small and constant amount of methaemoglobin and choleglobin.

1925; Brooks 1935), oxidation took place most rapidly at very low oxygen tensions which made the determination of the oxygen dissociation curves particularly difficult.

It has been suggested (Anson *et al.* 1924; Theorell 1934*a*, 1934*b*) that there is a linear relationship between the equilibrium constant $K = [\text{HbCO}] \times p\text{O}_2 / [\text{HbO}_2] \times p\text{CO}$ and the "span" such that $\log K/\text{span} = y$. The values for y obtained from vertebrate haemoglobins are shown in Table 5. The importance of these observations lies in the fact that because variations in K denote changes in the free energy of the system, displacement of the α -bands of oxy-haemoglobin and carboxyhaemoglobin would be accompanied by changes in the free energy. Keilin and Wang (1945, 1946) have already shown that the generalization does not hold with haemoglobin from legume root nodules and *Gastrophilus*. In the present work the value of y was found to be much lower than that for vertebrate haemoglobins (see Table 5) and it is clear that the argument advanced by Anson *et al.* (1924) also cannot be applied to the parasite haemoglobins.

TABLE 5
THE RELATIONSHIP BETWEEN THE "SPAN" AND THE PARTITION CONSTANT
 $K = [\text{HbCO}] \times p\text{O}_2 / [\text{HbO}_2] \times p\text{CO}$ OF SEVERAL DIFFERENT HAEMOGLOBINS

Origin of Haemoglobin	K	$y = \log K/\text{span}$	Observers
Blood of vertebrates	125-550	0.043-0.050	Anson <i>et al.</i> (1924)
Muscle of vertebrates	28-51	0.045-0.050	Roche (1932, 1933) Theorell (1934 <i>a</i> , 1934 <i>b</i>)
Root nodules of legumes	37	0.016	Keilin and Wang (1945)
<i>Gastrophilus</i> larvae	0.67	-0.0018	" " " (1946)
<i>Nematodirus</i> spp.	1.12 (mean of 4 values)	0.0008	Rogers (present paper)

It would appear that the properties of the nematode haemoglobins were such that the pigments might be expected to have considerable importance in the economy of the parasites, acting as oxygen acceptors at the low partial pressures which occur in the host gut fluid and carrying oxygen to cytochrome in the presence of cytochrome oxidase. However, before this matter can be discussed it will be necessary to know something of the behaviour of the haemoglobins in intact parasites. The results of such an investigation will be described in a later paper.

V. ACKNOWLEDGMENTS

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RUMENAL FLORA STUDIES IN THE SHEEP

I. THE NUTRITIVE VALUE OF RUMEN BACTERIAL PROTEIN

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Summary

Two large samples of "mixed" rumen bacteria, virtually free from protozoa and feed residues, were prepared from abattoir sheep, one from sheep coming from "green" feed conditions and the other from "dry"-fed sheep.

The "true" digestibilities and biological values of the crude protein of these preparations were determined by nitrogen-balance method with young growing rats and compared with "standard" casein. The cyst(e)ine and methionine contents of the rumen bacteria samples and of "whole" protein preparations made from them were also determined.

The average "true" digestibilities of the protein of the "green"-fed and "dry"-fed rumen bacteria were found to be 62.1 and 64.8 respectively. These were not significantly different from each other but very much lower than that obtained for the casein, namely 101.2.

The mean biological values found for the "dry"-fed rumen bacterial protein, the "green"-fed rumen bacterial protein, and the casein were 77.9, 79.9, and 79.6 respectively, when fed as the sole source of nitrogen in the ration at levels of 9.2, 9.7, and 9.5 per cent. crude protein ($N \times 6.25$).

The crude protein content of the "green"-fed bacterial sample was 47.6 per cent. and of the "dry"-fed 50.9 per cent. on the dry basis. These samples and their protein preparations were found to be very similar in cystine content in relation to the total nitrogen present, but the former was appreciably richer in methionine.

These findings were compared with those of other workers with similar material, with other microorganisms, and with other sources of protein, and are discussed in relation to the problem of the utilization of microbial protein by the ruminant.

It is concluded that judged by results with growing rats, rumen bacterial protein must be regarded as low in digestibility, relatively high in biological value, but mildly deficient in methionine.

I. INTRODUCTION

More than fifty years ago Zuntz (1891) and Hagemann (1891) postulated that the non-protein nitrogen of the diet might be converted to protein by the microorganisms of the rumen and that this bacterial protein could later be digested and utilized by the host animal. Since that time numerous investigators have established that protein synthesis by the bacteria of the rumen can and

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does occur. Indisputable evidence of such synthesis is provided by many experiments demonstrating the utilization of urea and ammonium salts by the ruminant. Extensive reviews of the early literature in this field are given by Mitchell and Hamilton (1929) and by Krebs (1937). The results of later studies up to 1947 are reviewed by McNaught and Smith (1947). Since that time further important data have been obtained* which show that the 10 essential amino acids can be synthesized in large amounts in ruminants fed a diet in which urea is virtually the sole source of dietary nitrogen.

Even where the dietary nitrogen is entirely in the form of protein, considerable alteration may be expected in satisfying the demands of the rumen bacteria. Johnson and his co-workers (1942) suggested that the conversion of food protein to bacterial protein was considerable. This view is strongly supported by the work of Hale, Duncan, and Huffman (1947), who showed a rumen "digestion" coefficient of 60 for protein, and more recently by McDonald (1948), who found that 40 per cent. of the protein zein fed to sheep was converted to microbial protein.

These findings emphasize the importance of knowing something of the nutritive value of rumen bacterial protein, a problem which was investigated by Müller (1906), Uselli and Fiorini (1938), and Johnson *et al.* (1944). Their results were reviewed by McNaught and Smith (*loc. cit.*), who also presented the results of an experiment carried out by McNaught *et al.* (1947) in which the true digestibility and biological value of the protein of a large preparation of "mixed" rumen bacteria were determined. The nutritive value of the rumen bacteria obtained by these workers was considerably higher than that obtained by Johnson *et al.* (*loc. cit.*), who had insufficient material to obtain entirely satisfactory results by the methods used.

In this paper are presented the methods and results of an investigation of the "true" digestibility, the biological value, and the sulphur-containing amino-acid contents of the protein of bacteria obtained from the rumen of slaughterhouse sheep selected to represent two types of feed conditions. The results of the sulphur-containing amino-acid determinations on these samples have been partially presented previously by Johanson, Moir, and Underwood (1949).

II. MATERIALS AND METHODS

(a) Collection of Rumen Bacteria

The samples of rumen bacteria were obtained from sheep slaughtered at the Melbourne Abattoirs. The rumens were separated into those from sheep coming from "dry" feed and those from "green" feed conditions. The absence of green material in the rumen was taken to indicate "dry"-fed animals and its presence to indicate "green"-fed animals.

* By Loosli, J. K., Williams, H. H., Thomas, W. E., Ferris, F. H., and Maynard, L. A. (1949), unpublished data.

The whole rumen contents were strained into cans through several thicknesses of gauze, transported to the laboratory, and immediately chilled in a refrigerated room. The materials were then again strained through gauze and centrifuged by means of a Sharples centrifuge, first at low speed (21,000 r.p.m.) to remove food particles, protozoa, and yeast cells, then at a higher speed (34,500 r.p.m.) to throw down the bacteria. This separation was incomplete, and approximately 20 per cent. of the smaller organisms passed through the centrifuge. Microscopic examination showed the solid material collected to be practically free of food particles. The bacterial sludge was spread out in shallow dishes and dried in a hot-air oven at 50-60°C. The resulting dry samples appeared as rather dark translucent flakes and were found to be virtually free from fibre. One hundred g. of material from the "dry"-fed and 90 g. from the "green"-fed sheep were prepared in this way. The chemical composition of these samples, together with that of other rumen bacteria preparations given in the literature, is given in Table 1.

TABLE 1
CHEMICAL COMPOSITION OF RUMEN BACTERIA SAMPLES

	Smith and Baker (1944) (%)	McNaught <i>et al.</i> (1947) (%)	Johnson <i>et al.</i> (1944) (%)	Reed, Moir, and Underwood (1949)	
				"Green" fed (%)	"Dry" fed (%)
Moisture	0.5	7.5	—	11.9	12.9
Ash	6.2	6.6	—	—	—
Crude Protein (N x 6.25)	36.3	41.1	44.5	41.9*	44.4†
Total carbohydrate	46.6	37.3	—	—	—
Ether extract	9.5	2.7	—	15.0	10.7
Crude fibre	—	<0.3	—	Trace	Trace

* Calculated to dry basis 47.6 per cent. crude protein.

† Calculated to dry basis 50.9 per cent. crude protein.

It is apparent that these two samples are very similar in protein content (N x 6.25) to those prepared by other workers but are appreciably higher in ether extract. The high value for the "green"-fed sample is no doubt influenced by the considerable quantities of ether-soluble pigments present, particularly chlorophyll.

Examination of the rumen samples prior to drying showed that there were some differences in the types of flora present. In the "dry"-fed samples coccid and oval cells predominated and the giant iodophile forms were frequently observed. In the "green"-fed samples there was a greater proportion of rod forms and a considerable increase in the spiral forms. There were no *Oscillospira* species in either sample and very few of only the smallest *Seletonas* species. The iodophile reaction was very indistinct in both samples, presumably because of the starved condition of the sheep before slaughter.

(b) *Experimental Methods*

The true digestibilities and biological values of the proteins were determined by the method of Mitchell (1923-4) as modified by Mitchell and Carmen (1926), Beadles *et al.* (1933), and Mitchell *et al.* (1945).

From 15 inbred male albino rats, two groups, each consisting of six rats, matched in pairs according to litter and body weight, were selected. The age of the rats was 6-7 weeks and their body weights ranged from 70 to 100 g. Each rat was housed separately in a metabolism cage which permitted accurate and separate collection of all urine and faeces voided. The animal room temperature was maintained at $23 \pm 1^\circ\text{C}$. for the entire experimental period. The rats were fed daily at 2 p.m. and always weighed 2-2½ hours after this time. Distilled water was supplied *ad libitum* and the food was fed as a creamy paste to avoid spilling and at a level to ensure a more or less equal consumption by all rats, with a minimum of food residues. Any such residues were collected and dried under the same conditions as for the moisture determinations in the original diet. Mitchell (*loc. cit.*) established that the percentage of nitrogen in the residues treated in this manner was identical with that of the ration from which they had been taken.

Urine and faeces were collected daily. All urine from 2 p.m. on the first collection day to the same time on the eighth day was collected to represent a seven-day collection period. To avoid loss of ammonia the collecting flasks contained 10 ml. of 1-2 per cent. conc. H_2SO_4 and the funnels, separating bulbs and flasks were washed daily with about 200 ml. of hot 1-2 per cent. H_2SO_4 . The whole of the urine and washings for each rat for each collection period was bulked and stored, with additional H_2SO_4 and a small amount of toluene, in the refrigerator. Before analysis it was filtered through glass wool, made up to standard volume, and the nitrogen determined on suitable aliquots by a slight modification of the micro-Kjeldahl method of Cole and Parkes (1946).

Ferric oxide and barium sulphate were used as faeces markers as recommended by Schneider (1935). The ferric oxide was fed (1 per cent. of diet) on the first and eighth collection days and the barium sulphate (1 per cent. of diet) in the intervening period. The faeces were collected to include all those stained by the first feeding of ferric oxide and the barium sulphate which followed, but did not include those stained by the second feeding of ferric oxide on the eighth day. The faeces of each rat were kept under H_2SO_4 in a desiccator, and the entire collection for each rat bulked, partially digested with H_2SO_4 , selenium, and K_2SO_4 , made up to standard volume, and aliquots taken for nitrogen determination as with the urines.

(c) *The Diets*

The composition of the various diets fed is given in Table 2. Sufficient was made up from the finely-ground dried constituents to supply the requirements of the whole experimental period. This was stored in the refrigerator when not in use.

With the exception of the low egg-N diet these diets were made up to contain between 9 and 10 per cent. of the test protein ($N \times 6.25$) and were isocaloric. The amounts of starch and butter fat were varied to allow for the amounts of test materials included and the caloric value of each was calculated as 4.7 cal. per g., of which 4.3 cal. per g. came from the carbohydrate and fat. This should have been sufficient to provide the energy requirements of the rat at the level consumed, independent of the protein supplied.

TABLE 2
COMPOSITION OF DIETS

	Low Egg-N		"Standard" Casein		Rumen Bacteria ("Dry" Fed)		Rumen Bacteria ("Green" Fed)	
	BaSO ₄	Fe ₂ O ₃	BaSO ₄	Fe ₂ O ₃	BaSO ₄	Fe ₂ O ₃	BaSO ₄	Fe ₂ O ₃
Whole dried egg, ether extracted	5	5	—	—	—	—	—	—
Casein	—	—	10.7	10.7	—	—	—	—
Rumen bacteria ("dry" fed)	—	—	—	—	20.2	20.2	—	—
Rumen bacteria ("green" fed)	—	—	—	—	—	—	21.4	21.4
Sugar	20	20	20	20	20	20	20	20
Butter fat	15	15	15	15	13	13	12	12
Cod-liver oil*	2	2	2	2	2	2	2	2
Salt mixture†	4	4	4	4	4	4	4	4
BaSO ₄	1	—	1	—	1	—	1	—
Fe ₂ O ₃	—	1	—	1	—	1	—	1
Starch	53	53	47.3	47.3	39.8	39.8	39.6	39.6
Total	100	100	100	100	100	100	100	100
Moisture	5.7	6.1	4.7	4.7	8.0	5.8	5.9	5.6
N (% on dry basis)	—	—	1.52	1.52	1.47	1.47	1.55	1.55
Protein ($N \times 6.25$)	—	—	9.50	9.50	9.19	9.19	9.69	9.69

* α -Tocopherol was added to the cod-liver oil at a level equivalent to that fed by Brosshardt and Barnes (1946).

† Steenbock No. 40 salt mixture.

A supplement of the water-soluble "B" vitamins* was made up from the diet used by Brosshardt and Barnes (1946), on the assumption that a rat consumed 10 g. of this diet daily. This was kept in a tightly stoppered dark bottle in the refrigerator and made up freshly every three weeks. Each day 0.25 ml. of the suspension was added to the food jar of each rat just prior to feeding. This ensured a constant daily vitamin intake by each rat despite variations in food intake.

(d) Order of Feeding

The rather limited amounts of dried rumen bacteria available made it impossible to employ an ideal design of experiment in which each sample

* The authors are indebted to Nicholas Pty. Ltd. for generous donations of these vitamins.

would be fed to each group of rats both before and after the low-nitrogen ration. Nevertheless, the use of a "standard" casein diet, for which ample data are available from the literature, on each group of rats, one before and one after the low-nitrogen ration, permitted a satisfactory design to be employed, as set out in Table 3.

TABLE 3
ORDER OF FEEDING OF DIETS

	First Period (10 days)	Second Period (14 days)	Third Period (9 days)
Group I (6 rats)	"Standard" casein diet	Low egg-N diet	Rumen bacteria diet ("dry" fed)
Group II (6 rats)	Rumen bacteria diet ("green" fed)	Low egg-N diet	"Standard" casein diet

The first period of 10 days was divided into a 3-day preliminary period, followed by a 7-day collection period. The second period included a 7-day preliminary period to allow the rats to reach a constant level of endogenous nitrogen excretion (Olson and Palmer 1940; Miller and Morrison 1942), followed by a 7-day collection period. In the third period, a 3-day preliminary period was followed by a 6-day collection period owing to shortage of material for Group 1.

(e) *Determination of the Sulphur-containing Amino Acids*

Representative 10-g. samples were taken from each of the dried bacterial preparations and ground to pass through a 0.5-mm. sieve. "Whole" protein preparations were made from the ground samples by the method of Lugg (1939), as modified by Lugg and Weller (1944). Nitrogen determinations were made on the ground samples and on the "whole" protein preparations by the micro-Kjeldahl method. Cyst(e)ine and methionine were determined by the differential oxidation method of Lugg (1938) together with the modified procedure of Lugg and Weller (loc. cit.) for total sulphur.

III. RESULTS

(a) *Nitrogen Balance Data*

The complete nitrogen balance data for each rat for each feeding period are presented in Appendix I. The mean "true" digestibilities and biological values of the test proteins, calculated from these data, are given in Table 4.

In calculating these results it is assumed that the metabolic faecal nitrogen per g. of dry matter consumed and the endogenous urinary nitrogen per 100 g. of body weight, obtained from the second feeding period on the low egg-N diet, were constant over the entire feeding trial. Such an assumption is supported by the data of Schneider (loc. cit.) and Smuts (1935).

It will be noted that the digestibility figures for the casein protein are slightly higher than the theoretically possible with both groups of rats. They are also slightly higher than the following values for this material given in the

literature: Beadles *et al.* (loc. cit.), 99.1; Olson and Palmer (loc. cit.) 99.7; Hughes and Hauge (1945), 100. This is due to a small negative balance of food nitrogen in the faecal nitrogen of the test periods. Similar, although much larger, negative balances are recorded in the data of Smuts and Marais (1938), Smuts and Malan (1938), and Marais and Smuts (1940). This phenomenon may be due to small cumulative errors in the experimental techniques or to the fact that the whole dried-egg protein used in the low-nitrogen diets may not have had a true digestibility of 100 as assumed, owing to its method of preparation or some other cause. In this work and in that of the South African workers just cited, the egg was dried in the air at 90-100°C., whereas it is probable that the American workers, who did not obtain any such negative balances in the food nitrogen of the faeces, used commercial egg powder, presumably dried at lower temperatures. It is possible also that the metabolic faecal nitrogen is not as constant from one feeding period to another as assumed. Some support for this possibility is provided by the data of Titus (1927) working with steers and of Brosshardt and Barnes (1946) working with mice.

TABLE 4
MEAN "TRUE" DIGESTIBILITIES AND BIOLOGICAL VALUES

	First Period		Second Period	Third Period	
	"True" Digestibility	Biological Value		"True" Digestibility	Biological Value
Group I (6 rats)	Casein 100.9 ± .28	76.8 ± 1.28	Low egg-N diet	Rumen bacteria 64.8 ± .03	("Dry" fed) 77.9 ± 1.88
Group II (6 rats)	Rumen bacteria 62.1 ± .64	("Green" fed) 79.9 ± 1.36		Casein 101.5 ± .51	82.5 ± .96

It will also be observed from Table 4 that the biological value of the casein is appreciably higher in the third feeding period, *after* the low-nitrogen period, than in the first feeding period. This difference is significant at the 1 per cent. level. Miller and Morrison (loc. cit.) also reported that the biological values for four rations following a low-nitrogen period were higher than the values obtained for the same rations with the same sheep before the low-nitrogen period. It is possible that there is an increased utilization of the absorbed nitrogen after a low-nitrogen period to replace the reserve protein stores of the liver and other tissues which have been depleted by the poor nitrogen intake. Ample evidence that the liver is readily depleted of a reserve protein store by a period of low nitrogen intake has been cited in the review of Kosterlitz and Campbell (1945), while French, Routh, and Mattill (1941) have observed that, when a short period of high protein follows a low-protein or non-protein régime, rats over-compensate by storing unusual amounts of protein. This point, together with that made in the previous paragraph, needs to be kept in mind

when considering the results for the rumen bacteria samples. It should also be mentioned that the mean value of 79.6 obtained for the biological value of the casein with all 12 rats is higher than the following values obtained under rather similar conditions of feeding by other workers: Beadles *et al.* (loc. cit.), 73; Hughes and Hauge (loc. cit.), 65; Olson and Palmer (loc. cit.), 62.

The outstanding finding revealed by the results given in Table 4 is the much lower digestibility found for the rumen bacterial protein than for casein. The mean "true" digestibilities of 62.1 for the "green"-fed sample and 64.8 for the "dry"-fed sample are not significantly different from each other but are both significantly lower than the mean value of 101.2 for the casein. Johnson *et al.* (loc. cit.), using a limited number of rats, found a still lower value for the true digestibility of the protein of mixed rumen bacteria, namely 55. McNaught *et al.* (loc. cit.), on the other hand, obtained a mean value of 72.3. This latter material differed from that described here and by Johnson *et al.* in that it was derived from incubated rumen material, and dried by the use of alcohol and ether, as compared with oven drying.

The position with respect to the biological values is quite different. Again there is no significant difference between the mean values obtained for the two rumen bacteria samples, but what the position would have been had the two samples been fed under identical conditions, i.e. in relation to the time of feeding the low-nitrogen ration, is not known. Possibly the "dry"-fed sample would have been significantly lower in biological value had it been fed before the low-nitrogen period, as with casein previously mentioned. The biological values of the protein of these samples of bacteria (77.9 and 79.9) are not significantly different, however, from the mean value obtained for the casein for the whole 12 rats (79.6), in which the time of feeding factor might be expected to be eliminated. These values lie between the very high mean figure of 88.4 obtained for the biological value of the protein of a similar rumen bacteria preparation by McNaught *et al.* (loc. cit.) fed at an 8 per cent. crude protein level, and the mean figure of 66 obtained by Johnson *et al.* (loc. cit.) using only 3 rats and feeding at an appreciably higher crude protein level (10.75 per cent.). This latter low value may be due, at least in part, to a less efficient utilization of the absorbed nitrogen at the higher level of protein intake, compared with a protein intake of 8 per cent. in the work of McNaught *et al.* (loc. cit.) and of 9.2 per cent. and 9.7 per cent. in the present investigation.

(b) "Whole" Protein Preparations

The moisture contents and the weights of total nitrogen, coagulable nitrogen, and protein nitrogen in the rumen bacteria samples, together with the nitrogen data for the "whole" protein preparations, are shown in Table 5.

It will be observed from Table 5 that, although the total nitrogen content of the bacteria from the sheep on "dry" feed is somewhat higher than that of the "green"-fed sample, the reverse is true of their protein separations. Both

bacterial samples contain considerable amounts of non-protein nitrogen, particularly the "green"-fed. During the "whole" protein preparations large quantities of extractable carbohydrates were removed from this latter sample, resulting in a preparation of high nitrogen content.

TABLE 5
NITROGEN DATA ON RUMEN BACTERIA SAMPLES AND THEIR "WHOLE" PROTEIN PREPARATIONS

Material	Fresh Wt. (g.)	Moisture (%)	Dry Wt. (g.)	Total N (% dry basis)	Total N (mg.)	Coag. N (mg.)	Protein N (mg.)	Dry Wt. "Protein" (g.)	N in "Protein" Prepara- tion (%)
Rumen bacteria "dry" fed	10.0	12.5	8.75	8.13	711	623	620	7.06	8.78
Rumen bacteria "green" fed	10.0	11.9	8.81	7.60	670	558	554	5.76	9.62

(c) Sulphur Distributions

In Table 6 the sulphur distributions of the "whole" proteins and of the unextracted bacteria samples are given. The values for cyst(e)ine and methionine nitrogen and the amounts of sulphate-S are in each case expressed as percentages of the total nitrogen present. Each value represents the mean of closely-agreeing duplicate estimations. The apparent losses of cyst(e)ine, methionine, and sulphate which occur in the extraction process are also shown. These losses must be qualified as "apparent" because of the possibility of the presence of other sulphur compounds in unextracted samples.

The results given in Table 6 indicate that the "dry"-fed sample has no extractable cyst(e)ine but some free methionine or other sulphur compounds present, whereas the "green"-fed sample contains a proportion of extractable cyst(e)ine and methionine or other sulphur compounds. It is apparent also that the two rumen bacteria samples and their protein preparations do not differ greatly in their contents of cyst(e)ine in relation to the total nitrogen present, although this is not true of their methionine contents. The "green"-fed sample of unextracted bacteria is appreciably higher in methionine than the "dry"-fed and the protein preparation from this "green"-fed sample is considerably higher.

It is not possible to state whether this difference is due to an effect of diet on the nature of the bacterial proteins, independent of the bacterial types, or to differences in the types of organism comprising the two mixed samples.

Many workers have shown that changes in the nature of the diet result in qualitative as well as quantitative changes in the rumen floral population (Quin 1943; Elsden 1945; Baker and Harris 1947; Moir, unpublished data 1949), while others have presented evidence pointing to a relatively constant amino-acid constitution of bacterial and yeast proteins over a wide range of growth conditions (Camien, Salle, and Dunn 1945; Block and Bolling 1945*a*; Freeland and Gale 1947). A comparison of the values given in Table 6 with those given

TABLE 6
PARTIAL COMPOSITION OF THE PREPARATIONS: LOSSES DURING EXTRACTION

No.	Material	Total N (% dry basis)	% of Total N		$\frac{\text{SO}_4\text{-S}}{\text{N}} \times 100$	Apparent Loss during Extraction		
			Cystine N	Methionine N		Cystine (%)	Methionine (%)	$\text{SO}_4\text{-S}$ (%)
1	Rumen bacteria "dry" fed	8.13	1.41	1.34	0.11	— 1	23	— 5
1p	"Whole" protein preparation of 1	8.78	1.63	1.18	0.13			
2	Rumen bacteria "green" fed	7.60	1.45	1.60	0.18	14	20	50
2p	"Whole" protein preparation of 2	9.62	1.50	1.54	0.12			

by Camien, Salle, and Dunn for four species of *Lactobacilli* and by Block and Bolling for eight strains of yeast indicates that the rumen bacterial protein is in each case twice as rich in cystine as the yeast protein and about twenty times as rich as the *L. fermenti* protein. These samples are also considerably richer in methionine than the protein of *Lactobacilli*, although not greatly different from yeast protein in this respect.

IV. DISCUSSION

It is clear from the results given in the preceding section that rumen bacterial protein is, compared with most sources of dietary protein, of high biological value but low digestibility. The biological values obtained for the

two samples were not significantly different from each other and not significantly different from such a recognized "quality" protein as casein, fed to the same rats under the same conditions. Their "true" digestibilities, however, were very much lower than that of casein. Direct comparison with the results of other investigators is impossible because of differences in the experimental conditions, but it is satisfactory to note that our values lie, in each case, between those of McNaught *et al.* (loc. cit.) and of Johnson *et al.* (loc. cit.). This is not unexpected in view of the fact that the former workers fed their material at an 8 per cent. crude protein level and the latter at a 10.75 per cent. crude protein level, compared with 9.2 and 9.7 per cent. in the present work, although it is not contended that these differences in protein level are wholly responsible for the differences in the results of the three investigations. These may also have been influenced to an unknown extent by differences in the types of bacteria present in the mixed samples.

The low digestibility obtained for the rumen bacterial protein in rats raises the question whether the ruminant animal is able to digest this protein any more successfully, and if so, whether the rumen protozoa normally play any part in the process. Johnson *et al.* (loc. cit.) found rumen protozoal protein to be very much more digestible in rats than bacterial protein from the same source, while the *in vitro* studies of Baker (1943) showed that protozoa from the rumen were readily accessible to peptic and tryptic digestion, but that the iodophile bacteria were resistant to digestion by pepsin and the effect of trypsin on these microorganisms varied with the type of organism and the brand of trypsin used. On the other hand, the former workers also showed that on medium levels of nitrogen intake, mostly in the form of urea, sheep freed of protozoa in their digestive tracts utilized the nitrogen of the rations to about the same extent as normal sheep. Clearly, much further research is necessary on this important aspect of nitrogen nutrition in the ruminant.

The relatively high biological values obtained for the rumen bacterial protein invite inquiry as to the quantitative significance of this type of protein in ruminant nutrition. Johnson and his colleagues (loc. cit.) have presented evidence indicating that up to a level of 10-12 per cent. crude protein ($N \times 6.25$) in the ration, which they regard as about the maximum amount of nitrogen capable of being handled by the rumenal microorganisms, a considerable proportion of the protein ultimately utilized by the ruminant is microbial protein, regardless of the nature of the dietary nitrogen. If this were true, all food nitrogen up to this level of intake should, as they suggest, exhibit a biological value characteristic of the nitrogen of the mixed microorganisms reaching the abomasum and duodenum. These workers go further and comment on the frequency with which biological values of about 60 have been obtained with ruminants fed a wide variety of rations containing 10-12 per cent. crude protein. The implication is that this figure represents the average biological value to young ruminants of this type of protein. The fact that in this investigation and in that of McNaught *et al.* (loc. cit.) biological values much higher than

60 have been obtained with rats for rumen bacterial protein does not necessarily invalidate the above theory because (i) the same protein may not have the same biological value for growth in lambs as for growth in rats (in fact, the results of Lofgreen, Loosli, and Maynard (1947) with whole egg protein indicate that they do not), and (ii) the role played by rumen protozoal protein and its biological value are largely unknown. Nor is this attractive theory necessarily invalidated by the results of Lofgreen, Loosli, and Maynard (*loc. cit.*), who found highly significant differences in the biological values of various sources of nitrogen when these were fed to lambs at a level equivalent to 10 per cent. crude protein. It must be pointed out that at present nothing is known of the possible differential effects of their rations on the numbers and types of rumenal flora and on the quantity and quality of the microbial protein formed. Some of these aspects of nitrogen nutrition in sheep are at present under investigation in this Laboratory.

The cystine and methionine values also call for further comment. Although, as pointed out in the preceding section, both samples of rumen bacteria were found to contain protein somewhat richer in methionine and very much richer in cystine than most microorganisms for which data are available, this does not mean that this protein is adequately supplied with sulphur-containing amino acids. Comparison with the values for "whole" egg protein given by Block and Mitchell (1946) shows that the rumen bacterial protein is very much lower in methionine and slightly lower in cystine than this almost perfectly utilizable protein, which has a biological value of nearly 100 for young growing rats. Comparison with the figures given by the same authors for casein shows that our rumen bacteria samples contain protein slightly lower in methionine but very much richer in cystine. Actual calculations, using the figures given in Table 6 and those of Block and Mitchell (*loc. cit.*) for casein, show that the casein diet fed to our rats supplied 29 mg. methionine-N and 3 mg. cystine-N per 10 g. of ration, whereas the "green"-fed rumen bacteria diet supplied 23 mg. methionine-N and 21 mg. cystine-N and the "dry"-fed rumen bacteria diet 19 mg. methionine-N and 20 mg. cystine-N per 100 g. of ration. Assuming that only one-sixth of the optimum methionine requirement of young growing rats can be supplied by cystine, as suggested by the experiments of Womack and Rose (1941), and in view of the fact that approximately equal amounts of the three diets were consumed by our rats, simple calculation shows that the level of intake of total "effective" sulphur-containing amino acids from the rumen bacterial protein and that from the casein were very similar.

These findings suggest that rumen bacterial protein is mildly deficient in sulphur-containing amino acids as has been found for casein by Beadles *et al.* (*loc. cit.*) and Kik (1938), and described by Block and Mitchell (*loc. cit.*). This conclusion is supported by the fact that the biological values of the protein of the two rumen bacteria samples were not significantly different from that of casein. This is in keeping with the data of Block and Bolling (1945*b*) for yeast protein, but differs from those of Klose and Fevold (1944) and Hock and Fink (1943).

It is true that these workers found yeast proteins to be primarily deficient in the sulphur-containing amino acids, but the former reported yeast to be seriously deficient in methionine only, relative to casein, while the latter found that cystine markedly improved its nutritive value. Moreover, our values for rumen bacterial protein are very considerably higher in cystine and appreciably higher in methionine than the *Lactobacillus fermenti* protein examined by Camien, Salle, and Dunn (loc. cit.). No doubt some of these differences are explainable in terms of the different methods of estimation used but, in addition, it is possible that the amino-acid constitution of the protein of different micro-organisms varies appreciably from type to type. Confirmation or otherwise of this possibility must await the results of further investigation. While the possible direct stimulating effect of added amino acid on the rumen population cannot be entirely neglected, it is suggested that on the present evidence the most reasonable explanation of the benefits obtained by adding methionine to urea diets in lambs (Loosli and Harris 1945; Lofgreen, Loosli, and Maynard loc. cit.) is that this amino acid supplements the microbial protein utilized by the host animal—protein in which methionine is the limiting amino acid.

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Rat No.	Initial Wt. (g.)	Final Wt. (g.)	Average Body Wt.* (g.)	Food Intake Dry Wt. (g.)	Food N Intake (mg.)	Faecal N (mg.)	Metabolic Faecal N (mg./g. food)	Metabolic Faecal N in Faeces (mg.)	Food N in Faeces (mg.)	Absorbed N (mg.)	Urinary N (mg.)
Casein diet - 1.500% nitrogen - 7-day collection period											
1	88.4	109.4	98.4	51.01	765.2	101.7	2.08	106.1	- 4.4	765.2	28.3
2	71.0	87.1	79.7	50.99	764.9	107.1	2.26	115.2	- 8.2	764.9	31.0
3	81.4	101.1	90.0	51.00	765.6	94.6	2.10	107.1	- 12.5	765.6	31.0
4	82.9	98.8	91.1	51.13	767.0	111.9	2.13	108.9	+ 2.0	767.0	31.0
5	72.0	85.8	80.2	51.06	765.9	105.1	2.27	115.9	- 10.9	765.9	31.0
6	82.5	95.6	90.7	51.12	766.8	103.5	2.16	110.4	- 6.9	766.8	31.0
"Green"-fed rumen flora diet - 1.55% nitrogen - 7-day collection period											
7	82.0	88.9	85.6	50.55	783.5	396.2	1.89	95.5	300.7	482.8	28.3
8	74.1	75.8	75.3	45.88	711.1	398.1	2.41	110.6	287.5	423.6	22.0
9	81.4	86.6	84.3	50.52	783.1	414.1	2.32	117.2	296.9	486.2	24.0
10	79.9	86.9	83.0	49.49	767.1	385.5	2.25	111.4	279.1	493.0	23.0
11	76.4	82.1	79.5	49.33	764.6	380.8	1.97	97.2	283.6	481.0	20.0
12	81.6	85.6	82.6	50.25	778.9	409.4	2.26	113.6	295.8	483.1	20.0
"Dry"-fed rumen flora diet - 1.47% nitrogen - 6-day collection period											
1	119.1	125.8	123.6	51.35	754.9	379.7	2.08	106.8	272.9	482.0	28.3
2	100.0	106.8	104.1	45.95	675.5	339.0	2.26	103.9	235.1	440.4	21.0
3	114.0	125.6	121.0	53.10	780.6	387.1	2.10	111.5	275.6	505.0	26.0
4	106.0	116.5	112.3	49.39	726.0	359.9	2.13	105.2	254.7	471.3	26.0
5	96.5	107.2	101.2	50.19	737.8	375.8	2.27	113.9	261.9	475.9	24.0
6	104.0	120.4	115.8	53.08	780.3	381.5	2.16	114.7	266.8	513.5	24.0
Casein diet - 1.500% nitrogen - 6-day collection period											
7	105.5	132.1	122.1	54.50	817.5	106.3	1.89	103.0	+ 3.3	817.5	30.0
8	87.5	106.6	100.0	54.39	815.9	104.5	2.41	131.1	- 26.6	815.9	30.0
9	100.8	119.5	112.0	54.49	817.4	117.0	2.32	126.4	- 9.4	817.4	29.0
10	105.5	127.7	117.9	54.45	816.8	101.2	2.25	122.5	- 21.3	816.8	30.0
11	97.8	120.4	109.3	54.45	816.8	104.4	1.97	107.3	- 2.8	816.8	29.0
12	104.6	122.2	116.2	52.68	790.2	100.0	2.26	119.1	- 19.1	790.2	27.0
Low-nitrogen period - 7-day collection period											
1	114.6	118.7	115.2	60.57		126.2	20.84				24.0
2	94.1	96.9	95.2	53.72		121.6	22.63				19.0
3	107.8	115.7	109.8	57.71		121.3	21.02				20.0
4	97.9	100.8	98.9	49.02		104.4	21.30				20.0
5	87.6	90.9	89.2	41.59		94.3	22.67				21.0
6	100.6	106.9	104.6	60.53		136.7	21.60				25.0
7	94.4	104.5	99.5	63.27		119.8	18.94				21.0
8	77.0	81.2	78.9	46.29		111.2	24.11				20.0
9	87.3	91.8	88.5	47.32		109.8	23.20				19.0
10	81.8	91.0	86.4	54.44		122.5	22.51				22.0
11	89.4	95.0	94.4	47.93		94.6	19.73				16.0
12	85.8	94.5	91.0	54.15		122.4	22.60				21.0

* Average of eight daily weighings.

IMPROVING THE ACCURACY OF GROWTH INDICES BY THE USE OF RATINGS

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Summary

A statistical procedure is developed whereby the precision of estimation of growth increments and various growth indices is greatly increased, especially where the variability of the plant material is great.

The procedure takes account of the fact that the difference between the mean weights of two successive harvests includes the difference between the sample means at the time of the first harvest. The importance of this factor is reduced by the use of ratings of both samples taken at the time of the first harvest. Weight comparisons are made by reference to the mean rating at this time or, where a succession of harvests is involved, to a suitable estimate of this mean rating.

The procedure is applied to a study on growth of tomatoes on a range of soil treatments and using simple chains of leaf area ratings. It is exemplified in detail from the control series of that experiment.

The data are examined critically to see whether they satisfy the assumptions inherent in the development of the theory. It is found that the variables of the bivariate distributions are highly correlated, with no evidence of a departure from a linear trend. Under these conditions, bias introduced from small departures from normality in the marginal distributions will be negligible.

Estimates of total weight, leaf weight, and leaf area based on maximum likelihood estimates of mean rating are more precise than are those based on mean rating at first harvest.

Gains in precision in estimates of relative growth rate and net assimilation rates are quite substantial, but there is little advantage in the use of maximum likelihood estimates in place of mean rating at first harvest for this purpose.

For estimates of weight, leaf area, and growth indices, the gain in information using ratings is as great for the absolute as it is for the logarithmic data.

General considerations relevant to the application of the procedure are discussed, and its merits and limitations are indicated.

I. INTRODUCTION

The conventional procedure for determining the increments in total dry weights, leaf weights, etc. of a plant species growing under a specific set of conditions is to make a succession of harvests of random samples, determine the means, and from these to estimate the growth increments and various growth indices. The difference between the mean weights of two harvests

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involves not only the sampling variation in the increments for individual plants between the two harvests but also the deviation of the mean of the later harvested plants from the other set at the time of the first harvest. As the interval between harvests is shortened this latter factor becomes progressively more important until in the limit of zero increment it is the sole source of error.

The importance of this factor can be reduced if it is possible to make objective measurements or ratings* which do not harm the plants on both sets at the time of the first harvest, these measurements being highly correlated with total weights, leaf weight, etc. Comparisons between weights at the harvests may then be made at the mean rating, the sampling error of which is only of importance if the regression slopes of weight on rating at the two harvests diverge. The sampling error of the mean rating can be reduced if plants not harvested on either occasion are also rated at the first harvest. More than one rating could be used but the increase in computational labour would rarely justify this extension.

II. RATING FOR A SUCCESSION OF HARVESTS

This principle of making comparisons over any interval by reference to a mean rating at the beginning of the interval leads directly to a variety of possible methods of arranging ratings for a succession of harvests. One of the simplest is to rate at the beginning of each interval only the sets of plants to be harvested at the beginning and end of the interval. Excluding the first and last harvests all plants for intermediate harvests are rated twice, at the beginning of the interval and at the end immediately preceding harvest.

For such intermediate harvests we may then have for each plant the initial and final ratings and the final leaf and total weights. If these measures can be regarded as distributed in a multivariate normal distribution, and this applies to the measures for each successive sample, and, further, if the variances and covariances for each of these distributions are known, one can readily estimate by the method of maximum likelihood or by least squares the population means and error of estimate for each measure at each harvest, and also any function of these parameters such as growth indices, differences in growth indices over successive intervals, etc.

In practice the population variances and covariances are not known. The errors of estimate with sample variances and covariances substituted for the corresponding population values will in consequence understate the true error by an amount which would be very laborious to estimate in any instance. For this and other reasons, including computational simplicity in developing and solving the normal equations, this approach has been confined in the subsequent treatment to improved estimates of only the mean rating at each harvest and it will be convenient to outline the procedure at this point.

* The presence of large subjective errors is not necessarily to be inferred from the use of this term. In general, the more objective the rating the better.

As an example consider the simple chain outlined above with only three harvests (Fig. 1), where \bar{x} is the mean rating of a set of plants and the subscript indicates the interval and whether the measure is made at the beginning (*b*) or end (*e*). Suppose the number of cases associated with \bar{x}_{01e} , \bar{x}_{12b} , and \bar{x}_{23b} are n_1 , n_2 , and n_3 respectively. Let the population mean ratings at harvests 1 and 2

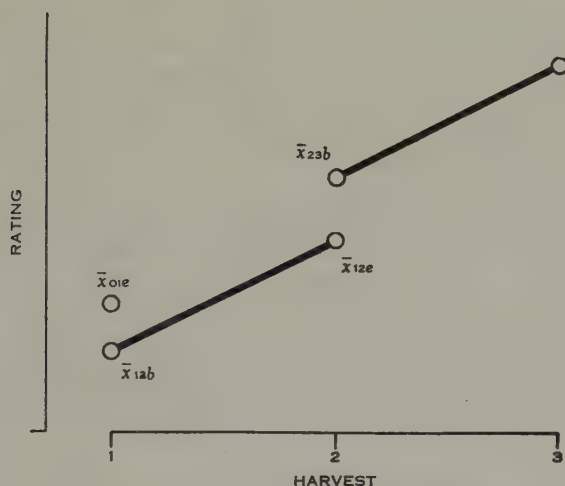


Fig. 1

be μ_{10} and μ_{01} , the variances μ_{20} and μ_{02} , the covariance μ_{11} and the correlation coefficient ρ . Then the joint sampling distribution of the sample mean ratings is

$$\text{constant} \times \exp. -\frac{1}{2} \left[\frac{n_1(\bar{x}_{01e} - \mu_{10})^2}{\mu_{20}} + \frac{n_2}{1 - \rho^2} \left\{ \frac{(\bar{x}_{12b} - \mu_{10})^2}{\mu_{20}} - \frac{2\mu_{11}(\bar{x}_{12b} - \mu_{10})(\bar{x}_{12e} - \mu_{01})}{\mu_{20}\mu_{02}} + \frac{(\bar{x}_{12e} - \mu_{01})^2}{\mu_{02}} \right\} + \frac{n_3(\bar{x}_{23b} - \mu_{01})^2}{\mu_{02}} \right] d\bar{x}_{01e} d\bar{x}_{12b} d\bar{x}_{12e} d\bar{x}_{23b} \dots (1)$$

Minimizing the logarithm for μ_{10} and μ_{01} gives as normal equations

$$\frac{n_1(\bar{x}_{01e} - \mu_{10})}{\mu_{20}} + \frac{n_2}{1 - \rho^2} \left\{ \frac{(\bar{x}_{12b} - \mu_{10})}{\mu_{20}} - \frac{\mu_{11}(\bar{x}_{12e} - \mu_{01})}{\mu_{20}\mu_{02}} \right\} = 0,$$

$$\frac{n_2}{1 - \rho^2} \left\{ -\frac{\mu_{11}(\bar{x}_{12b} - \mu_{10})}{\mu_{20}\mu_{02}} + \frac{(\bar{x}_{12e} - \mu_{01})}{\mu_{02}} \right\} + \frac{n_3(\bar{x}_{23b} - \mu_{01})}{\mu_{02}} = 0,$$

which have as solution for μ_{10} ,

$$\mu_{10} = \{ (n_1 + n_2)(n_2 + n_3)\mu_{20}\mu_{02} - n_1n_3\mu_{11}^2 \}^{-1} [n_2n_3(\bar{x}_{23b} - \bar{x}_{12e})\mu_{11}\mu_{20} + n_2(n_2 + n_3)\bar{x}_{12b}\mu_{20}\mu_{02} + n_1\bar{x}_{01e} \{ (n_2 + n_3)\mu_{20}\mu_{02} - n_3\mu_{11}^2 \}].$$

It will be noted that the sum of the coefficients of \bar{x}_{12b} and \bar{x}_{01e} equals unity while the coefficients of \bar{x}_{23b} and \bar{x}_{12e} are equal and opposite so that the expectation is unbiased. Similar relations hold of course for μ_{01} . The usual method

of numerical solution would be to form the reciprocal matrix of the coefficients of μ_{10} and μ_{01} . Then the estimate of μ_{10} is

$$c_{11} \left\{ \frac{n_1 \bar{x}_{01e}}{\mu_{20}} + \frac{n_2}{1 - \rho^2} \left(\frac{x_{12b}}{\mu_{20}} - \frac{\mu_{11} \bar{x}_{12e}}{\mu_{20} \mu_{02}} \right) \right\} \\ + c_{12} \left\{ \frac{n_2}{1 - \rho^2} \left(- \frac{\mu_{11} \bar{x}_{12b}}{\mu_{20} \mu_{02}} + \frac{\bar{x}_{12e}}{\mu_{02}} \right) + \frac{n_3 \bar{x}_{23b}}{\mu_{02}} \right\} \dots (2)$$

The collected coefficients of \bar{x}_{01e} , \bar{x}_{12b} , and \bar{x}_{23b} should then identically satisfy the above relations. If sample estimates of μ_{20} , μ_{11} , and μ_{02} are used, and there will be different estimates of μ_{20} , for example, from x_{01e} and x_{12b} , the same relations will apply as these differences can be absorbed in the n_1, n_2, n_3 multipliers. The error of the unbiased estimate as given by the c matrix will, however, tend to underestimate the true error.

An alternative approach to the estimate of μ_{10} is to determine the weighted mean with least error of the estimates given by \bar{x}_{01e} , \bar{x}_{12b} and $\bar{x}_{12b} + \bar{x}_{23b} - \bar{x}_{12e}$. The variances and covariances of these estimates can be estimated and hence the best weighted mean. This will give the same solution as the method outlined above. Other measures which are less efficient but unbiased can be developed along the same lines. Thus we may take $\frac{1}{2}(\bar{x}_{01e} + \bar{x}_{12b})$ as one estimate and $\bar{x}_{12b} + \bar{x}_{23b} - \bar{x}_{12e}$ as another and determine the best weighted mean of these.

Either approach, through normal equations or weighted means, will provide estimates of the population means and their sampling errors but the latter will be biased because the errors in the weights, that is, in the variances and covariances, are ignored.

III. IMPROVED ESTIMATE OF POPULATION MEAN

Consider now the use of ratings to improve the estimates of the population mean weights, etc. In the subscript notation of the following sections, the number 1 will refer to plants in a first interval and 2 to plants in the following interval, both sets of plants being rated at the harvest separating the two intervals. As before, measures at the beginning and end of an interval will be denoted by (b) and (e) respectively.

Assuming that weights and ratings are bivariate normal variables then between y_{1e} and x_{1e} for example we have a regression relation of the form

$$y = \bar{y}_{1e} + b_1(x - \bar{x}_{1e}),$$

where \bar{y}_{1e} and \bar{x}_{1e} are the mean weights and ratings of n_1 plants. If \hat{x} is the mean of these n_1 and $N - n_1$ additional ratings at the same time then

$$\hat{y} = \bar{y}_{1e} + b_1(\hat{x} - \bar{x}_{1e}) \dots (3)$$

has an expectation equal to the population mean ε and the expectation of $(\hat{y} - \varepsilon)^2$ is

$$\frac{\sigma^2 y_{1e} - \beta_1^2 \sigma^2 x_{1e}}{n_1} + \sigma^2 b_1 \left(\frac{\sigma^2 x_{1e}}{n_1} - \frac{\sigma^2 x_{1e}}{N} \right) + \beta_1^2 \frac{\sigma^2 x_{1e}}{N}, \dots (4)$$

the components of which may be referred to as array variation, slope variation and reference point variation respectively, β_1 being the parameter of which b_1 is an estimate. Denoting

$$\frac{\sum (y - \bar{y}_{1e})^2 - b_1^2 \sum (x - \bar{x}_{1e})^2}{n_1 - 2} \text{ as } S^2_{y_{1e} \cdot x_{1e}}$$

an unbiased estimator of this variance is

$$\frac{S^2_{y_{1e} \cdot x_{1e}}}{n_1} + \frac{S^2_{y_{1e} \cdot x_{1e}}}{n_1 - 3} \left(\frac{1}{n_1} - \frac{1}{N} \right) + \frac{1}{N} \left(\frac{b_1^2 \sum (x - \bar{x}_{1e})^2}{n_1 - 2} - \frac{\sum (y - \bar{y}_{1e})^2}{(n_1 - 1)(n_1 - 2)} \right),$$

which can be alternatively written

$$S^2_{y_{1e} \cdot x_{1e}} \left\{ \frac{n_1 - 2}{n_1(n_1 - 3)} - \frac{2n_1 - 4}{N(n_1 - 1)(n_1 - 3)} \right\} + \frac{b_1^2 \sum (x - \bar{x}_{1e})^2}{N(n_1 - 1)} \quad (5)$$

If for \hat{x} we had substituted the maximum likelihood estimate for the rating at this harvest with variance $S^2_{\hat{x}}$, an approximate estimate of the variance of the estimate \hat{y}_{1e} is given by

$$\begin{aligned} & S^2_{y_{1e} \cdot x_{1e}} \left\{ \frac{n_1 - 2}{n_1(n_1 - 3)} - \frac{2S^2_{\hat{x}}}{\sum (x - \bar{x}_{1e})^2} \right\} + b_1^2 S^2_{\hat{x}} \\ & + 2b_1 \left\{ \frac{\sum (y - \bar{y}_{1e})(x - \bar{x}_{1b}) - b_1 \sum (x - \bar{x}_{1e})(x - \bar{x}_{1b})}{n_1(n_1 - 2)} \times \text{coefficient of } \bar{x}_{1b} \text{ in the} \right. \\ & \quad \left. \text{estimate of } \hat{x} \right\} \quad \dots \dots \dots (6) \end{aligned}$$

The last term arises from the contribution of \bar{x}_{1b} to the estimate \hat{x} and the correlation of \bar{x}_{1e} and \bar{y}_{1e} with it. A convenient condensed notation for the factor multiplying $2b_1$ is $[y_{1e}]$. The corresponding factor of $2b_2$ in the estimate of the variance of \hat{y}_{2e} referred to x_{2b} is

$$\frac{\sum (y - \bar{y}_{2e})(x - \bar{x}_{2e}) - b_2 \sum (x - \bar{x}_{2b})(x - \bar{x}_{2e})}{n_2(n_2 - 2)} \times \text{coefficient of } \bar{x}_{2e} \text{ in the estimate}$$

of \hat{x} and would be designated $[y_{2e}]$.

IV. IMPROVED ESTIMATE OF MEAN INCREMENT

Suppose now we have two normal bivariate populations which have a common marginal distribution of one of the variables x and samples of size n_1 and n_2 are drawn from them. In general the slopes of the regression lines and the dispersions about the lines for the two distributions will be different. The estimated difference of the mean y variates for the two populations at a reference point \hat{x} is

$$\hat{y}_{2e} - \hat{y}_{1e} = \bar{y}_{2e} - \bar{y}_{1e} + b_2(\hat{x} - \bar{x}_{2b}) - b_1(\hat{x} - \bar{x}_{1e}) \quad \dots (7)$$

If \hat{x} is the mean of the n_1 and n_2 observations and a further n_3 observations on the x variate alone, when $n_1 + n_2 + n_3 = N$, then for repeated samplings of the three sets of observations the expected value of $\hat{y}_{2e} - \hat{y}_{1e}$ is the difference in the population means for the variates, $\epsilon_2 - \epsilon_1$, and the expectation of

$$\{(\hat{y}_{2e} - \hat{y}_{1e}) - (\varepsilon_2 - \varepsilon_1)\}^2 \text{ is}$$

$$\frac{\sigma^2_{y_{2e} \cdot x_{2b}}}{n_2} + \frac{\sigma^2_{y_{1e} \cdot x_{1e}}}{n_1} + \sigma^2_{b_2} \left(\frac{\sigma^2_x}{n_2} - \frac{\sigma^2_x}{N} \right) + \sigma^2_{b_1} \left(\frac{\sigma^2_x}{n_1} - \frac{\sigma^2_x}{N} \right)$$

$$+ (\beta_2 - \beta_1)^2 \frac{\sigma^2_x}{N}, \dots \dots \dots (8)$$

where $\sigma^2_{x_{1e}} = \sigma^2_{x_{2b}} = \sigma^2_x$.

An unbiased estimator of this is

$$S^2_{y_{2e} \cdot x_{2b}} \left\{ \frac{n_2 - 2}{n_2(n_2 - 3)} - \frac{2n_2 - 4}{N(n_2 - 1)(n_2 - 3)} \right\} + S^2_{y_{1e} \cdot x_{1e}} \left\{ \frac{n_1 - 2}{n_1(n_1 - 3)} \right.$$

$$\left. - \frac{2n_1 - 4}{N(n_1 - 1)(n_1 - 3)} \right\} + \frac{1}{N} \left\{ b_2^2 \frac{\Sigma(x - \bar{x}_{2b})^2}{n_2 - 1} \right.$$

$$\left. - 2b_2 b_1 \left(\frac{\Sigma(x - \bar{x}_{2b})^2 \Sigma(x - \bar{x}_{1e})^2}{(n_2 - 3/2)(n_1 - 3/2)} \right)^{\frac{1}{2}} + b_1^2 \frac{\Sigma(x - \bar{x}_{1e})^2}{n_1 - 1} \right\}. \dots (9)$$

If for \hat{x} the maximum likelihood estimate is used, an estimate of the variance of $\hat{y}_2 - \hat{y}_1$ is given by

$$S^2_{y_{2e} \cdot x_{2b}} \left\{ \frac{n_2 - 2}{n_2(n_2 - 3)} - \frac{2S^2_{\hat{x}}}{\Sigma(x - \bar{x}_{2b})^2} \right\} + S^2_{y_{1e} \cdot x_{1e}} \left\{ \frac{n_1 - 2}{n_1(n_1 - 3)} \right.$$

$$\left. - \frac{2S^2_{\hat{x}}}{\Sigma(x - \bar{x}_{1e})^2} \right\} + (b_2 - b_1)^2 S^2_{\hat{x}} + 2(b_2 - b_1) \{[y_{2e}] - [y_{1e}]\}. \dots (10)$$

The effect on the form of the expression for the variance as a result of extending from one dependent variable to two is obvious and further extension to more complex expressions than $\hat{y}_2 - \hat{y}_1$ involves no particular difficulties. The only additional issue raised is in the case of an expression of the form $f(y_1, y_2)$ where y_1 and y_2 are two measures of the same plant which are correlated and which are referred to the same set of ratings. In this instance the elements of array variation are not independent and their covariance can be expressed in the form $\sigma_{y_2 \cdot x} \sigma_{y_1 \cdot x} \rho_{y_2 y_1 \cdot x}$ where $\rho_{y_2 y_1 \cdot x}$ is the partial correlation of y_1 and y_2 holding x constant. Similarly the covariance of the elements of slope variation is $\sigma_{b_{y_2 x}} \sigma_{b_{y_1 x}} \rho_{y_2 y_1 \cdot x}$.

V. APPLICATION TO DERIVED GROWTH INDICES

Consider now the application of the foregoing to growth data and derived measures such as relative growth rate and net assimilation rate. Weights of whole plants or tops of whole plants will be indicated by W , weights of leaves by LW , and leaf areas by LA . Used as ratings the leaf areas will be designated M with the mean of N simultaneous ratings or the maximum likelihood estimate taken over successive links in the chain by \hat{M} . The subscript notation is the same as for Section IV above.

Typical expressions for the estimate of weight are

$$\hat{W}_{1e} = \bar{W}_{1e} + b_{W_{1e}M_{1e}} (\hat{M} - M_{1e}), \quad \dots \quad (11)$$

and for the variance of estimate by substitution in (4), thus

$$\sigma^2 \hat{W}_{1e} = \sigma^2 \bar{W}_{1e.M_{1e}} + \sigma^2 b_{W_{1e}M_{1e}} (\sigma^2 \bar{M}_{1e} - \sigma^2 \hat{M}) + \beta^2_{W_{1e}M_{1e}} \sigma^2 \hat{M} \dots \quad (12)$$

Using for mean rating the maximum likelihood estimate, the corresponding variance from (6) is

$$S^2_{W_{1e}.M_{1e}} \left(\frac{n_1 - 2}{n_1(n_1 - 3)} - \frac{2S^2 \hat{M}}{\Sigma (M - \bar{M}_{1e})^2} \right) + b^2_{W_{1e}M_{1e}} S^2 \hat{M} \\ + 2b_{W_{1e}M_{1e}} [W_{1e}]. \quad \dots \quad (13)$$

If logarithms (to base 10) of all measures have been used instead of the actual values the expression for $S^2 \hat{W}_{1e}$ in logarithmic units will be as above. If now the antilog of \hat{W}_{1e} be determined the corresponding variance to a good approximation will be given by

$$(\text{Antilog } \hat{W}_{1e})^2 \{ S^2 \hat{W}_{1e} (\log_e 10)^2 + 3 S^4 \hat{W}_{1e} (\log_e 10)^4 \}, \quad \dots \quad (14)$$

and in general the term involving $S^4 \hat{W}_{1e}$ can be ignored.

(a) *The Variance of the Relative Growth Rate*, $R = \frac{\log_e (\bar{W}_{2e}/\bar{W}_{1e})}{t_2 - t_1}$

(i) *Without Use of Ratings*.—Arithmetic mean values are entered into the expression for R . The ratio of mean values will not be the same as the mean of the ratios for individual plants which of course cannot be determined, but will not differ substantially when changes over the interval are approximately constant multiples of the corresponding initial values for all plants in the treatment.

In the development of this and subsequent expressions it will be assumed that coefficients of variation are sufficiently small to justify the approximate methods employed.

The change in R corresponding to small changes $d\bar{W}_{2e}$ and $d\bar{W}_{1e}$ is $\frac{1}{t_2 - t_1} \left\{ \frac{d\bar{W}_{2e}}{\bar{W}_{2e}} - \frac{d\bar{W}_{1e}}{\bar{W}_{1e}} \right\}$ so that approximately the variance of R is estimated by

$$\frac{1}{(t_2 - t_1)^2} \left\{ \frac{\sigma^2 \bar{W}_{2e}}{\bar{W}_{2e}^2} + \frac{\sigma^2 \bar{W}_{1e}}{\bar{W}_{1e}^2} \right\} \dots \quad (15)$$

(ii) *With Use of Ratings*.— $R = \frac{\log_e (\hat{W}_{2e}/\hat{W}_{1e})}{t_2 - t_1}$. With ratings the varia-

tion in W_2 , for example, when referred to a mean of N simultaneous ratings may be regarded as

$$\Delta(\text{array } \bar{W}_{2e}) + (\bar{M}_{2b} - \hat{M}) \Delta b_{W_{2e}M_{2b}} + \Delta \hat{M} \beta_{W_{2e}M_{2b}},$$

and the variation in R as

$$\frac{1}{t_2 - t_1} [\Delta(\text{array } \bar{W}_{2e}) + (\bar{M}_{2b} - \hat{M})\Delta b_{W_{2e}M_{2b}} + \Delta \hat{M}\beta_{W_{2e}M_{2b}}]1/\hat{W}_{2e} \\ - \{\Delta(\text{array } \bar{W}_{1e}) + (\bar{M}_{1e} - \hat{M})\Delta b_{W_{1e}M_{1e}} + \Delta \hat{M}\beta_{W_{1e}M_{1e}}\}1/\hat{W}_{1e}].$$

The required variance is then

$$\frac{1}{(t_2 - t_1)^2} \left[\left\{ \sigma^2 \bar{W}_{2e}M_{2b} + (\sigma^2 \bar{M}_{2b} - \sigma^2 \hat{M})\sigma^2 b_{W_{2e}M_{2b}} \right\} \frac{1}{\hat{W}_{2e}^2} \right. \\ \left. + \left\{ \sigma^2 \bar{W}_{1e}M_{1e} + (\sigma^2 \bar{M}_{1e} - \sigma^2 \hat{M})\sigma^2 b_{W_{1e}M_{1e}} \right\} \frac{1}{\hat{W}_{1e}^2} \right. \\ \left. + \sigma^2 \hat{M} \left(\frac{\beta_{W_{2e}M_{2b}}}{\hat{W}_{2e}} - \frac{\beta_{W_{1e}M_{1e}}}{\hat{W}_{1e}} \right)^2 \right]. \dots \dots (16)$$

The estimator in the case where there are N simultaneous observations at the reference harvest is obvious from preceding samples. Using the maximum likelihood reference rating the estimator is approximately

$$\frac{1}{(t_2 - t_1)^2} \left\{ \frac{S^2 W_{2e}M_{2b}}{\hat{W}_{2e}^2} \left(\frac{n_2 - 2}{n_2(n_2 - 3)} - \frac{2S^2 \hat{M}}{\Sigma(M - \bar{M}_{2b})^2} \right) \right. \\ \left. + \frac{S^2 W_{1e}M_{1e}}{\hat{W}_{1e}^2} \left(\frac{n_1 - 2}{n_1(n_1 - 3)} - \frac{2S^2 \hat{M}}{\Sigma(M - \bar{M}_{1e})^2} \right) + S^2 \hat{M} \left(\frac{b_{W_{2e}M_{2b}}}{\hat{W}_{2e}} - \frac{b_{W_{1e}M_{1e}}}{\hat{W}_{1e}} \right)^2 \right. \\ \left. + 2 \left(\frac{b_{W_{2e}M_{2b}}}{\hat{W}_{2e}} - \frac{b_{W_{1e}M_{1e}}}{\hat{W}_{1e}} \right) \left(\frac{[W_{2e}]}{\hat{W}_{2e}} - \frac{[W_{1e}]}{\hat{W}_{1e}} \right) \right\}. \dots (17)$$

(iii) *Logarithms Without Use of Ratings.*—If logs of leaf area ratings and of total weights have been used throughout, write $\log_e W_{2e} = U_{2e}$, $\log W_{1e} = U_{1e}$. Then the variance of R is

$$\frac{1}{(t_2 - t_1)^2} (\sigma^2 U_{2e} + \sigma^2 U_{1e}). \dots \dots \dots (18)$$

If logs to base 10 have been used this is to be multiplied by $(\log_e 10)^2$.

(iv) *Logarithms With Use of Ratings.**—The variance of R is approximately

$$\frac{1}{(t_2 - t_1)^2} \left\{ \sigma^2 \bar{U}_{2e}M_{2b} + (\sigma^2 \bar{M}_{2b} - \sigma^2 \hat{M})\sigma^2 b_{U_{2e}M_{2b}} + \sigma^2 \bar{U}_{1e}M_{1e} \right. \\ \left. + (\sigma^2 \bar{M}_{1e} - \sigma^2 \hat{M})\sigma^2 b_{U_{1e}M_{1e}} + \sigma^2 \hat{M} (\beta_{U_{2e}M_{2b}} - \beta_{U_{1e}M_{1e}})^2 \right\}. \dots (19)$$

* The same symbols are used for log ratings as for ratings.

(b) *The Variance of the Net Assimilation Rate,*

$$E_{LW} = \frac{\overline{W}_{2e} - \overline{W}_{1e}}{\overline{LW}_{2e} - \overline{LW}_{1e}} \frac{\log_e(LW_{2e}/LW_{1e})}{t_2 - t_1}$$

(i) *Without Use of Ratings.*—The change in E_{LW} corresponding to small changes in estimated mean weights and leaf weights, $d\overline{W}_{2e}$, $d\overline{W}_{1e}$, $d\overline{LW}_{2e}$, and $d\overline{LW}_{1e}$ is

$$\frac{1}{(t_2 - t_1)(\overline{LW}_{2e} - \overline{LW}_{1e})} \left\{ (d\overline{W}_{2e} - d\overline{W}_{1e})A + d\overline{LW}_{2e}B - d\overline{LW}_{1e}C \right\}$$

where $A = \log_e(\overline{LW}_{2e}/\overline{LW}_{1e})$,

$$B = \frac{\overline{W}_{2e} - \overline{W}_{1e}}{\overline{LW}_{2e}} - \frac{\overline{W}_{2e} - \overline{W}_{1e}}{\overline{LW}_{2e} - \overline{LW}_{1e}} \log_e(\overline{LW}_{2e}/\overline{LW}_{1e}),$$

$$C = \frac{\overline{W}_{2e} - \overline{W}_{1e}}{\overline{LW}_{1e}} - \frac{\overline{W}_{2e} - \overline{W}_{1e}}{\overline{LW}_{2e} - \overline{LW}_{1e}} \log_e(\overline{LW}_{2e}/\overline{LW}_{1e}).$$

Then the variance of E_{LW} is

$$\frac{1}{(t_2 - t_1)^2 (\overline{LW}_{2e} - \overline{LW}_{1e})^2} \left\{ A^2 \sigma^2 \overline{W}_{2e} + 2AB \sigma \overline{W}_{2e} \sigma \overline{LW}_{2e} \rho_{W_{2e} LW_{2e}} + B^2 \sigma^2 \overline{LW}_{2e} \right. \\ \left. + A^2 \sigma^2 \overline{W}_{1e} + 2AC \sigma \overline{W}_{1e} \sigma \overline{LW}_{1e} \rho_{W_{1e} LW_{1e}} + C^2 \sigma^2 \overline{LW}_{1e} \right\} \dots (20)$$

(ii) *With Use of Ratings.*— W_{2e} and LW_{2e} are referred to a common rating scale and similarly for W_{1e} and LW_{1e} . The change in E corresponding to changes possible in \hat{W}_{2e} , \hat{W}_{1e} , \hat{LW}_{2e} , and \hat{LW}_{1e} can be symbolized by

$$\left[\{ \Delta(\text{array } \overline{W}_{2e}) + (\overline{M}_{2b} - \hat{M}) \Delta b_{W_{2e} M_{2b}} + \Delta \hat{M} \beta_{W_{2e} M_{2b}} \} A \right. \\ - \{ \Delta(\text{array } \overline{W}_{1e}) + (\overline{M}_{1e} - \hat{M}) \Delta b_{W_{1e} M_{1e}} + \Delta \hat{M} \beta_{W_{1e} M_{1e}} \} A \\ + \{ \Delta(\text{array } \overline{LW}_{2e}) + (\overline{M}_{2b} - \hat{M}) \Delta b_{LW_{2e} M_{2b}} + \Delta \hat{M} \beta_{LW_{2e} M_{2b}} \} B \\ \left. - \{ \Delta(\text{array } \overline{LW}_{1e}) + (\overline{M}_{1e} - \hat{M}) \Delta b_{LW_{1e} M_{1e}} + \Delta \hat{M} \beta_{LW_{1e} M_{1e}} \} C \right],$$

divided by $(t_2 - t_1)(\hat{LW}_{2e} - \hat{LW}_{1e})$,

where A , B , C are as given previously but with the regression estimates of \hat{W}_{2e} etc. substituted for \overline{W}_{2e} etc.

The approximate variance of E_{LW} is then

$$\left[A^2 \sigma^2 \overline{W}_{2e} M_{2b} + 2AB \sigma \overline{W}_{2e} M_{2b} \sigma \overline{LW}_{2e} M_{2b} \rho_{W_{2e} LW_{2e} M_{2b}} + B^2 \sigma^2 \overline{LW}_{2e} M_{2b} \right. \\ \left. + \{ A^2 \sigma^2 b_{W_{2e} M_{2b}} + 2AB \sigma b_{W_{2e} M_{2b}} \sigma b_{LW_{2e} M_{2b}} \rho_{W_{2e} LW_{2e} M_{2b}} + B^2 \sigma^2 b_{LW_{2e} M_{2b}} \} \right] \\ \times (\sigma^2 \overline{M}_{2b} - \sigma^2 \hat{M})$$

$$\begin{aligned}
& + A^2\sigma^2\overline{W}_{1c.M_{1c}} + 2AC\sigma\overline{W}_{1c.M_{1c}}\sigma\overline{LW}_{1c.M_{1c}}\rho W_{1c}LW_{1c.M_{1c}} + C^2\sigma^2\overline{LW}_{1c.M_{1c}} \\
& + \left\{ A^2\sigma^2b_{W_{1c}M_{1e}} + 2AC\sigma b_{W_{1c}M_{1e}}\sigma b_{LW_{1c}M_{1c}}\rho W_{1c}LW_{1c.M_{1e}} + C^2\sigma^2b_{LW_{1c}M_{1e}} \right\} \\
& \quad \times (\sigma^2\overline{M}_{1e} - \sigma^2\hat{M}) \\
& + \sigma^2\hat{M} \left\{ A(\beta_{W_{2c}M_{2e}} - \beta_{W_{1c}M_{1e}}) + B\beta_{LW_{2c}M_{2e}} - C\beta_{LW_{1c}M_{1e}} \right\}^2 \Big] \\
& \quad \text{divided by } (t_2 - t_1)^2(\hat{LW}_{2e} - \hat{LW}_{1e})^2. \quad \dots \dots (21)
\end{aligned}$$

In practice, $\sigma\overline{W}_{2c.M_{2b}}\sigma\overline{LW}_{2c.M_{2b}}\rho W_{2c}LW_{2c.M_{2b}}$ would be estimated as

$$\frac{1}{n_2(n_2 - 2)} \left\{ \Sigma W_{2e}LW_{2e} - \frac{\Sigma W_{2e}M_{2b}\Sigma LW_{2e}M_{2b}}{\Sigma M_{2b}^2} \right\} \text{ i.e. } \frac{\Sigma W_{2e}LW_{2e}M_{2b}}{n_2},$$

where squares and products refer to deviates from sample means. For the maximum likelihood value of \hat{M} , the estimator would be

$$\begin{aligned}
& \left[\left(A^2S^2W_{2c.M_{2b}} + 2ABS_{W_{2e}LW_{2c.M_{2b}}} + B^2S^2LW_{2c.M_{2b}} \right) \right. \\
& \quad \times \left(\frac{n_2 - 2}{n_2(n_2 - 3)} - \frac{2S^2\hat{M}}{\Sigma(M - \overline{M}_{2b})^2} \right) \\
& + \left(A^2S^2W_{1c.M_{1e}} + 2ACS_{W_{1c}LW_{1c.M_{1e}}} + C^2S^2LW_{1c.M_{1e}} \right) \\
& \quad \times \left(\frac{n_1 - 2}{n_1(n_1 - 3)} - \frac{2S^2\hat{M}}{\Sigma(M - \overline{M}_{1e})^2} \right) \\
& + S^2\hat{M} \left\{ A(b_{W_{2c}M_{2b}} - b_{W_{1c}M_{1e}}) + Bb_{LW_{2c}M_{2b}} - Cb_{LW_{1c}M_{1e}} \right\}^2 \\
& + 2 \left\{ A(b_{W_{2c}M_{2b}} - b_{W_{1c}M_{1e}}) + Bb_{LW_{2c}M_{2b}} - Cb_{LW_{1c}M_{1e}} \right\} \\
& \quad \left. \left\{ A([W_{2e}] - [W_{1e}]) + B[LW_{2e}] - C[LW_{1e}] \right\}^2 \right] \\
& \quad \text{divided by } (t_2 - t_1)^2(\hat{LW}_{2e} - \hat{LW}_{1e})^2. \quad \dots \dots (22)
\end{aligned}$$

(iii) *Logarithms Without Use of Ratings.*—If logarithms have been used throughout for total weights, leaf weights, and leaf areas, and we write

$$\begin{aligned}
\log_e W_{2e} &= U_{2e}, & \log_e W_{1e} &= U_{1e}, \\
\log_e LW_{2e} &= V_{2e}, & \log_e LW_{1e} &= V_{1e}; \\
\text{then } E_{LW} &= \frac{e^{\overline{U}_{2e}} - e^{\overline{U}_{1e}}}{e^{\overline{V}_{2e}} - e^{\overline{V}_{1e}}} \cdot \frac{\overline{V}_{2e} - \overline{V}_{1e}}{t_2 - t_1},
\end{aligned}$$

geometric means being substituted for arithmetic means in Section (i).

The change in E_{LW} corresponding to small changes in $d\bar{U}_{2e}$, $d\bar{U}_{1e}$, $d\bar{V}_{2e}$ and $d\bar{V}_{1e}$ is

$$\frac{1}{(t_2 - t_1)(e^{\bar{V}_{2e}} - e^{\bar{V}_{1e}})} \left[d\bar{U}_{2e} e^{\bar{U}_{2e}} (\bar{V}_{2e} - \bar{V}_{1e}) - d\bar{U}_{1e} e^{\bar{U}_{1e}} (\bar{V}_{2e} - \bar{V}_{1e}) \right. \\ \left. + d\bar{V}_{2e} e^{\bar{V}_{2e}} \left\{ \frac{e^{\bar{U}_{2e}} - e^{\bar{U}_{1e}}}{e^{\bar{V}_{2e}}} - \frac{e^{\bar{U}_{2e}} - e^{\bar{U}_{1e}}}{e^{\bar{V}_{2e}} - e^{\bar{V}_{1e}}} (\bar{V}_{2e} - \bar{V}_{1e}) \right\} \right. \\ \left. + d\bar{V}_{1e} e^{\bar{V}_{1e}} \left\{ \frac{e^{\bar{U}_{2e}} - e^{\bar{U}_{1e}}}{e^{\bar{V}_{1e}}} - \frac{e^{\bar{U}_{2e}} - e^{\bar{U}_{1e}}}{e^{\bar{V}_{2e}} - e^{\bar{V}_{1e}}} (\bar{V}_{2e} - \bar{V}_{1e}) \right\} \right],$$

which may be written

$$\left[d\bar{U}_{2e}(\bar{W}_{2e}A) - d\bar{U}_{1e}(\bar{W}_{1e}A) + d\bar{V}_{2e}(\bar{LW}_{2e}B) - d\bar{V}_{1e}(\bar{LW}_{1e}C) \right]$$

divided by $(t_2 - t_1)(\bar{LW}_{2e} - \bar{LW}_{1e})$.

The expression in Section (i) will then apply if $(\bar{W}_{2e}A)$ is substituted for A , to be used in conjunction with the variation in \bar{U}_{2e} and so on. The A , B , C values of this section are of course based on geometric means.

(iv) *Logarithms With Use of Ratings.*—The development follows along the same lines as in Section (ii) with the substitutions for A etc. as given in Section (iii).

(c) *The Variance of the Net Assimilation Rate,*

$$E_{LA} = \frac{\bar{W}_{2e} - \bar{W}_{1e}}{\bar{LA}_{2e} - \bar{LA}_{1e}} \frac{\log_e(\bar{LA}_{2e}/\bar{LA}_{1e})}{t_2 - t_1}$$

(i) *Without Use of Ratings.*—The required variance can be obtained from (20), substituting LA for LW .

(ii) *With Use of Ratings.*—In this case leaf area is being used in a dual role.

$\beta_{LA_{1e}M_{1e}}$ and $b_{LA_{1e}M_{1e}}$ each equals one, while $\sigma^2_{b_{LA_{1e}M_{1e}}}$, $\sigma^2_{\bar{LA}_{1e}M_{1e}}$, and $\rho_{W_{1e}LA_{1e}M_{1e}}$ are zero. $[LA_{1e}]$ is also zero.

The required variance and maximum likelihood estimator can be obtained by making the obvious substitutions in (21) and (22).

(iii and iv) *Logarithms Without and With Use of Ratings.*—These follow along the same lines as (iii), (iv) of the previous section, with obvious substitutions.

VI. APPLICATION OF THEORY TO A SPECIFIC SET OF DATA

(a) *Experimental and General*

In a study on growth of tomatoes on a range of soil treatments, the procedure outlined here was followed for a simple chain of leaf-area ratings. A series of photographic standards was used to estimate the areas of individual leaves and hence the leaf area for the whole plant. For the first and second harvests, very large numbers of plants were available and these were sampled thoroughly for each of the treatments. For each sample the leaf blades, including cotyledonary leaves, were separated from the rest of the shoots, and dry weights were obtained for each fraction separately.

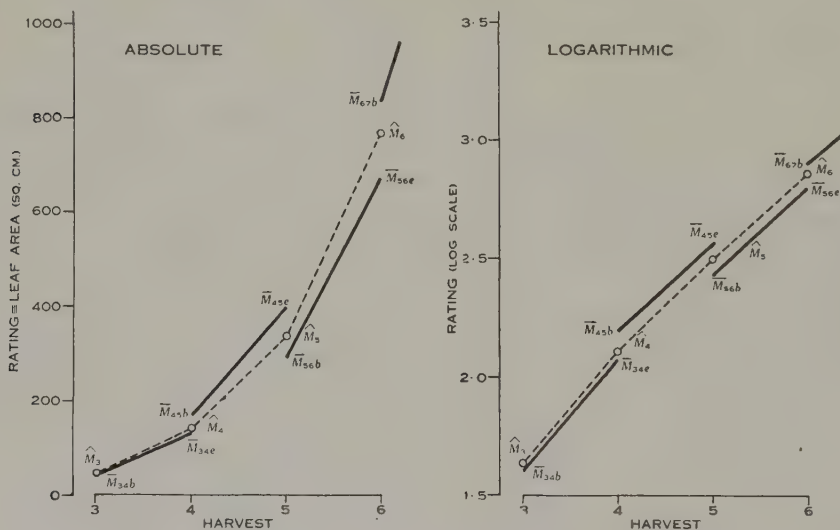


Fig. 2.—Absolute and logarithmic ratings (leaf areas) plotted against time. For plants of harvest 4, \bar{M}_{34b} is the mean rating at the beginning of harvest interval 3-4, and \bar{M}_{34e} is the mean rating taken at the end of the interval and just prior to harvesting the sample; \hat{M}_4 is the maximum likelihood estimate for the rating at harvest 4, and so on. The mean rating \bar{M}_{23e} is omitted from this figure.

For harvest 3 and within each treatment, three sets of sixteen plants were taken at random and rated for leaf area. One of these sets was harvested immediately* and the dry weights of the leaf blades and total shoots determined as before.

For harvest 4, the second of these sets was again rated for leaf area and then* harvested. The third set of sixteen plants had been rated as an insurance against casualties among plants of the second group. At the time of re-rating the second set of plants, a further set of sixteen plants (including all remaining spares of the harvest 3 rating) was rated together with eight instead of sixteen plants.

* Actually, the following day in each case.

The general procedure for harvest 4 was repeated for harvests 5 and 6. At harvest 7 no estimates of leaf area were made. The first five harvest intervals were 7 days and the last interval was 14 days.

The successive ratings of the above procedure formed a chain as illustrated in Figure 2 from the absolute and logarithmic data of the control series. The picture is a simple extension of the specific case of Section II above, though the notation is slightly different. The mean rating of a set of plants is now \bar{M} ; as before, the subscript indicates the harvest interval and whether the rating is made at the beginning (*b*) or end (*e*) of the interval. The broken lines of Figure 2 join the maximum likelihood estimates, \hat{M}_3 - \hat{M}_6 of the population mean ratings for successive harvests. These give inter-harvest trends which are closely parallel to the experimental trends established by the individual links of the chain.

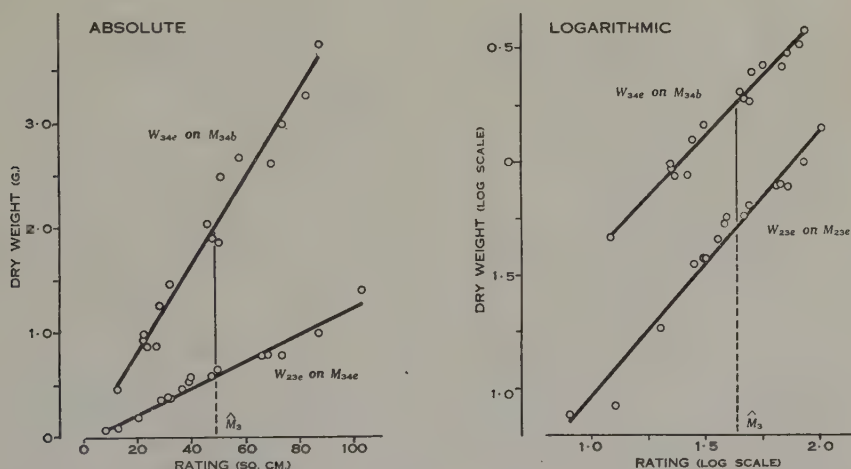


Fig. 3.—The relations between dry weight of plants (tops only) at harvests 3 and 4 and their ratings (leaf areas) at the commencement of harvest interval 3-4. The pairs of regression lines are linked by the maximum likelihood estimates, \hat{M}_3 of the true rating as ordinate.

The weight-rating relation (bivariate distribution) is illustrated in Figures 3-6 for the successive harvest intervals of the control series. In each case the absolute and logarithmic data are shown side by side for comparison. In preparing the figures, the log scales were kept constant throughout, but the absolute scales were scaled up or down so as to give regressions which were readily comparable with the corresponding logarithmic regressions. The weight-area scale-ratio was kept constant for all four sets of absolute data. In Figure 3, the dry weights of the plants (tops only) for harvests 3 and 4 are plotted against their leaf areas at the commencement of the interval. The two groups of sixteen weight values are highly correlated with their leaf areas, and the relations are adequately described by the linear regressions.

$$W_{23e} = -.02379 + .012575 M_{23e},$$

$$W_{34e} = .00724 + .041194 M_{34b},$$

and

$$\log_{10} W_{23e} = -2.2091 + 1.1687 \log_{10} M_{23e},$$

$$\log_{10} W_{34e} = -1.4490 + 1.0378 \log_{10} M_{34b}.$$

It is obvious that the departures of individual values from the regressions are trifling by comparison with their departures from the means of their marginal distributions, and it is mainly upon this fact that the whole procedure depends for the improvement of the accuracy of plant weights and growth indices.

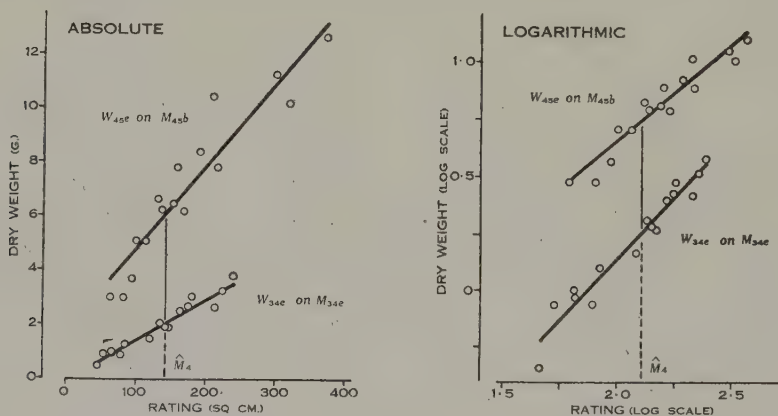


Fig. 4.—The relations between dry weight of plants (tops only) at harvests 4 and 5 and their ratings (leaf areas) at the commencement of harvest interval 4-5. The pairs of regression lines are linked by the maximum likelihood estimates, \hat{M}_4 of the true rating as ordinate.

The linear regressions of Figures 4-6 are as follows:

Figure 4

$$W_{34e} = -.13048 + .015202 M_{34e},$$

$$W_{45e} = 1.74395 + .030703 M_{45b},$$

and

$$\log_{10} W_{34e} = -2.0411 + 1.0880 \log_{10} M_{34e},$$

$$\log_{10} W_{45e} = -1.0245 + .8394 \log_{10} M_{45b}.$$

Figure 5

$$W_{45e} = -.08766 + .018185 M_{45e},$$

$$W_{56e} = .29022 + .038189 M_{56b},$$

and

$$\log_{10} W_{45e} = -1.8427 + 1.0364 \log_{10} M_{45e},$$

$$\log_{10} W_{56e} = -1.3644 + .9828 \log_{10} M_{56b}.$$

Figure 6

$$W_{56e} = -.42254 + .017777 M_{56e},$$

$$W_{67e} = 49.3623 + .027836 M_{67b},$$

and

$$\log_{10} W_{56e} = -1.9931 + 1.0792 \log_{10} M_{56e},$$

$$\log_{10} W_{67e} = .9952 + .2979 \log_{10} M_{67b}.$$

Estimates of population mean weights and hence of the mean increments from harvest to harvest are given by substituting the values of the estimates

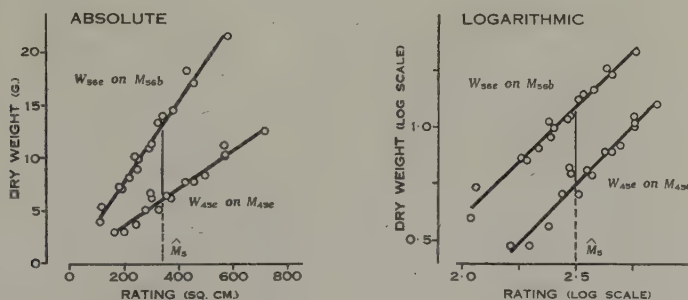


Fig. 5.—The relations between dry weight of plants (tops only) at harvests 5 and 6 and their ratings (leaf areas) at the commencement of harvest interval 5-6. The pairs of regression lines are linked by the maximum likelihood estimates, \hat{M}_5 of the true rating as ordinate.

of the mean ratings (see Fig. 2 and Table 4) in the regression equations. Before proceeding to do this, however, it is necessary to examine the data more critically, for it is essential to the strict application of the theory that the assumptions inherent in its development should be satisfied by the data.

(b) Tests of Normality and Linearity

The primary assumption of the development is that the variables are random samples from bivariate or multivariate normal distributions, and this implies marginal normal distributions, linear regressions, and uniform array variability. From inspection of the plotted data (Figs. 3-6) one would infer that these conditions are not seriously violated. Statistical tests of normality and linearity have been restricted to the control treatment, but there is little reason to believe that these results will not be representative of the other treatments as well. For the significance of departure from normality, the usual tests of asymmetry (Kendall 1946) and kurtosis (Geary and Pearson 1938) have been employed and the results are set out in Table 1.

The thirteen variables listed in Table 1 are not all independent; in fact, the maximum number of independent values in a set is five. In only one case, W_{23e} (logs), is the asymmetry significant but there is a suggestion of positive

asymmetry (n.s.) in the absolute values and negative asymmetry ($P < 0.05$) in the logarithmic values. This applies whether one considers the five distributions of weight or the corresponding leaf area distributions. The only individual

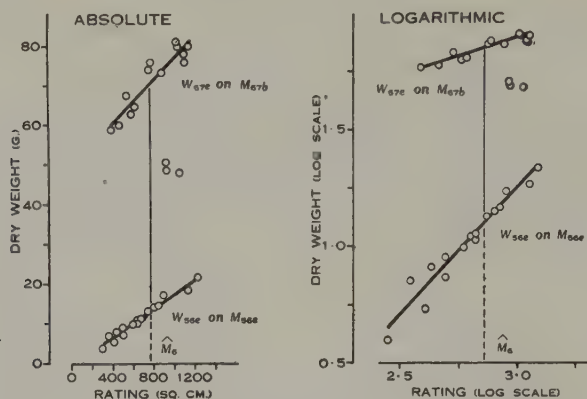


Fig. 6.—The relations between dry weight of plants (tops only) at harvests 6 and 7 and their ratings (leaf areas) at the commencement of harvest interval 6-7. The pairs of regression lines are linked by the maximum likelihood estimates, \hat{M}_6 of the true rating as ordinate. The three aberrant values for harvest 7 are not included in the regressions for that harvest (see text).

departures of kurtosis from expectation which are exceptional are associated with the thirteen plants of interval 6-7. Taken over sets of independent distributions the mean departures are not significant.

TABLE 1
TESTS OF NORMALITY

Absolute					Logarithmic				
Variable	n	Asymmetry		Kurtosis	Variable	n	Asymmetry		Kurtosis
		$t = k_3/k_2^{3/2}$	σ_t				$t = k_3/k_2^{3/2}$	σ_t	
				$a = \frac{M.D.}{S.D.}$					$a = \frac{M.D.}{S.D.}$
W_{23e}	16	0.7621	0.5643	0.7672	W_{23e}	16	-1.1047	0.5643	0.7625
W_{34e}	16	0.3277	0.5643	0.8540	W_{34e}	16	-0.5305	0.5643	0.8638
W_{45e}	16	0.2359	0.5643	0.8303	W_{45e}	16	-0.4337	0.5643	0.7995
W_{56e}	16	0.5280	0.5643	0.8152	W_{56e}	16	-0.4481	0.5643	0.7999
W_{67e}	13	-0.4917	0.5979	0.8962	W_{67e}	13	-0.5645	0.5979	0.8949
LA_{23e}	16	0.6451	0.5643	0.8502	LA_{23e}	16	-0.7769	0.5643	0.7661
LA_{34b}	16	0.4065	0.5643	0.8450	LA_{34b}	16	-0.3856	0.5643	0.8763
LA_{43e}	16	0.1747	0.5643	0.8581	LA_{34e}	16	-0.4070	0.5643	0.8821
LA_{45b}	16	0.9555	0.5643	0.8011	LA_{45b}	16	-0.0318	0.5643	0.8075
LA_{45e}	16	0.4446	0.5643	0.8535	LA_{45e}	16	-0.2607	0.5643	0.8358
LA_{56b}	16	0.6837	0.5643	0.7905	LA_{56b}	16	-0.4153	0.5643	0.7960
LA_{56e}	16	0.6848	0.5643	0.7945	LA_{56e}	16	-0.1556	0.5643	0.8186
LA_{67b}	13	-0.1153	0.5979	0.8953	LA_{67b}	13	-0.4967	0.5979	0.8660

In the test of linearity (Table 2) all pairs of variables which have been examined are recorded. There is no reason to believe that they constitute a selection which is in any sense biased. In general, the quadratic term is not

TABLE 2
TESTS OF LINEARITY

Variables	Absolute				Logarithmic			
	Linear Regression Coefficient	Linear Residual Variance	Variance due to Quadratic Term	Correlation Coefficient	Linear Regression Coefficient	Linear Residual Variance	Variance due to Quadratic Term	Correlation Coefficient
LA_{34e} on LA_{34b}	2.6256	189	454	0.9776	0.9180	0.00292	0.00012	0.9744
LA_{45e} on LA_{45b}	1.6569	2823	7211	0.9445	0.7785	0.00369	0.00113	0.9457
LA_{56e} on LA_{56b}	2.0334	7074	82	0.9531	0.8387	0.00427	0.00676	0.9353
W_{23e} on LA_{23e}	0.0126	0.00448	0.00034	0.9819	1.1687	0.00484	0.01045	0.9817
W_{34e} on LA_{34b}	0.0412	0.03757	0.02170	0.9818	1.0378	0.00229	0.00196	0.9840
W_{45e} on LA_{45e}	0.0181	0.40406	0.10426	0.9773	1.0364	0.00222	0.00035	0.9716
W_{56e} on LA_{56b}	0.0382	0.56633	0.56094	0.9888	0.9828	0.00110	0.00002	0.9868
W_{67e} on LA_{67b}	0.0278	9.50690	29.30765	0.9288	0.2979	0.00029	0.00001	0.9441
LW_{23e} on LA_{23e}	0.0088	0.00214	0.00007	0.9824	1.1555	0.00468	0.01329	0.9819
LW_{34e} on LA_{34b}	0.0281	0.01614	0.00806	0.9831	1.0240	0.00208	0.00133	0.9851
LW_{45e} on LA_{45e}	0.0104	0.15012	0.04040	0.9746	0.9470	0.00206	0.00033	0.9686
LW_{56e} on LA_{56b}	0.0202	0.41616	1.13903	0.9714	0.9225	0.00162	0.00002	0.9782
LW_{67e} on LA_{67b}	0.0140	2.61052	3.29875	0.9230	0.5071	0.00120	0.00000	0.9241

significant for either the absolute or logarithmic values. Because of this, the residual variance after removing only the variance due to the linear term has been given.

Homogeneity of array variance has not been examined statistically but from inspection it appears that for logarithmic data the arrays associated with low ratings are more variable than those associated with high ratings. The converse may apply to the absolute data.

Summarizing, the variables are highly correlated with no evidence of departure from a linear trend. There is a tendency to negative asymmetry in the marginal distributions for logarithmic values and possibly greater array variability associated with lower ratings. It is certain that, under conditions of linearity and high correlation, bias introduced from small departure from normality in the marginal distributions will be negligible, so that there is little objection to the application of normal distribution theory to this data. Preferably one would consider only the absolute values but for purposes of illustration and contrast the logarithmic values have also been used.

Several points of interest emerge from an examination of the bivariate distributions of Figures 3-6. The regressions of weight (or leaf weight) on leaf area of samples at two successive harvests referred to leaf area at the first harvest tend to diverge markedly for the absolute data, the regression coeffi-

cients often being as much as three times as great at the end as at the beginning of the interval. With the logarithmic data, however, the tendency is reversed, though the regressions are much more nearly parallel (e.g. Figs. 3 and 5). Then, too, the residual variance is much greater for the second than for the first harvest of each pair on the absolute basis, and it tends to be rather less on the logarithmic basis. These trends are confirmed for the other treatments and must be regarded as real for the stages of growth covered by the experiment. In consequence, the much simpler variance expressions which would result on the basis of parallel slopes and equal array variance are not permissible here (see Goodall 1945).

The data for harvest interval 6-7 (Fig. 6) differ from the rest in that the interval was fourteen instead of seven days. All lateral shoots had been nipped out at an early stage up to the time of harvest 6, but this procedure was neglected during a period of wet weather just after this harvest. In consequence, there was a "flush" growth of upper laterals which could not be removed because of their contribution to the weight increment. It is probable that this "flush" growth was more pronounced in the smaller plants within each treatment, and that this helped to bring about the near-parallelism of the regressions for the absolute data and the very pronounced convergence of the regressions for the logarithmic data.

Finally, it will be noted that three values for harvest 7 (Fig. 6) are far short of the weights predicted by the regression for the remaining thirteen values. It is believed, though there are no specific records to confirm it, that these discrepancies are due to a genetically controlled character causing "blindness" of the apical meristem. A number of plants, irrespective of treatment, had shown this condition and had been rejected accordingly. However, in the presence of the "flush" growth mentioned above, it is likely that the fault was overlooked in the three plants in question. In all, six such aberrant values were detected for harvest 7 of the experiment. The phenomenon is of interest in itself, but its detection in this way points to the value of the rating technique for the detection and, where justifiable, the rejection of such aberrant values.

(c) Calculation of Maximum Likelihood Estimates of the Mean Ratings

The expression for the likelihood (equation 1) was extended to include additional links in the chain, and the normal equations were formed by differentiating with respect to the four required mean parameters, M_3 , M_4 , M_5 , and M_6 . These equations were solved using the reciprocal matrix method. The coefficients of the separate sample means contributing to the estimates of mean ratings at the four harvests (see Table 3) satisfy the requirements for unbiased estimates.

In Table 4 are given the separate mean ratings of the same harvest date, the means of all ratings at this date (means of 32), and the maximum likelihood estimate. As might be expected, the variance of the mean of all ratings

on a given harvest date is approximately half of those of the separate mean ratings. The maximum likelihood estimate, which takes into account the linkage with the ratings of the remaining three harvests, carries with it a further reduction in the variance. The relation of the maximum likelihood estimate to the ratings of the separate samples is indicated in Figure 2, and the improvement of the estimates of the means is evident from their smooth time trends.

TABLE 3

COEFFICIENTS OF THE SEPARATE SAMPLE MEANS CONTRIBUTING TO THE MAXIMUM LIKELIHOOD ESTIMATES OF THE MEAN RATINGS

Sample Mean	Absolute				Logarithmic			
	\hat{M}_3	\hat{M}_4	\hat{M}_5	\hat{M}_6	\hat{M}_3	\hat{M}_4	\hat{M}_5	\hat{M}_6
\bar{M}_{23e}	0.2474	0.6088	0.7615	1.3997	0.1793	0.1457	0.0961	0.0683
\bar{M}_{34b}	0.7526	-0.6088	-0.7615	-1.3997	0.8207	-0.1457	-0.0961	-0.0683
\bar{M}_{34e}	-0.1652	0.5305	0.6635	1.2196	-0.6112	0.3884	0.2563	0.1821
\bar{M}_{45b}	0.1652	0.4695	-0.6635	-1.2196	0.6112	0.6116	-0.2563	-0.1821
\bar{M}_{45e}	-0.0668	-0.1899	0.5178	0.9518	-0.4421	-0.4424	0.5557	0.3949
\bar{M}_{56b}	0.0668	0.1899	0.4822	-0.9518	0.4421	0.4424	0.4443	-0.3949
\bar{M}_{56e}	-0.0160	-0.0456	-0.1157	0.6912	-0.2721	-0.2722	-0.2734	0.6530
\bar{M}_{67e}	0.0160	0.0456	0.1157	0.3088	0.2721	0.2722	0.2734	0.3470

(d) *Improved Estimates of Total Weight, Leaf Weight, and Leaf Area*

By using the additional information available on the population mean rating at each harvest it is now possible to make improved estimates of total weight, leaf weight, and leaf area. This is done, as already indicated for total weight, by substituting the estimates of the mean ratings in the regression equations of Section (a) above. These estimates of weight are given in Table 5 and also graphically for maximum likelihood estimates of mean rating in Figures 3-6. In Figure 7, also, all regressions for the logarithmic data are combined in one diagram, and the estimates of weight are shown for the maximum likelihood estimates of mean rating.

The ratio of the variance of the unadjusted to adjusted values cannot exceed the ratio of the total information on the rating to the information from the bivariate distribution alone. The advantage of the extra information in maximum likelihood estimates relative to mean rating at first harvest is here apparent.

The variance of differences of \hat{W} in different treatments is the sum of the corresponding variances. The appropriate variance for differences of \hat{W} from successive harvests of the same treatment is given by substitution in (9) or (10). For differences of \hat{W} from the same treatment but not from successive harvests one can choose reference points which are not correlated if using mean

TABLE 4
A. RATINGS = LEAF AREA (ABSOLUTE VALUES)

Harvest	Experimental Values			Estimates of True Rating			Maximum Likelihood Estimate	
	Rating of Harvested Sample		Rating of Next Harvest Sample	Mean of all Ratings at Harvest		\hat{M}_3 \hat{M}_4 \hat{M}_5 \hat{M}_6	Variance	
	Mean	Variance		Mean	Variance		Mean	Variance
3	M_{23e} 43.33	44.51	M_{34b} 45.86	\bar{M}_3 46.09	19.13	\hat{M}_3	48.47	11.01
4	M_{34e} 133.33	249.34	M_{45b} 174.63	\bar{M}_4 153.98	194.25	\hat{M}_4	140.71	77.01
5	M_{45e} 395.56	1526.31	M_{56b} 291.63	\bar{M}_5 343.59	692.05	\hat{M}_5	337.30	244.56
6	M_{56e} 666.56	4509.50	M_{67b} 829.81	\bar{M}_6 748.18	2245.52	\hat{M}_6	766.19	1199.43

B. RATINGS = LEAF AREA (LOGARITHMIC VALUES)

Harvest	Experimental Values			Estimates of True Rating			Maximum Likelihood Estimate	
	Rating of Harvested Sample		Rating of Next Harvest Sample	Mean of all Ratings at Harvest		\hat{M}_3 \hat{M}_4 \hat{M}_5 \hat{M}_6	Variance	
	Mean	Variance		Mean	Variance		Mean	Variance
3	M_{23e} 1.5850	0.005500	M_{34b} 1.6013	\bar{M}_3 1.5932	0.002252	\hat{M}_3	1.6388	0.000986
4	M_{34e} 2.0715	0.003372	M_{45b} 2.1907	\bar{M}_4 2.1311	0.001656	\hat{M}_4	2.1095	0.000802
5	M_{45e} 2.5637	0.002033	M_{56b} 2.4246	\bar{M}_5 2.4942	0.001246	\hat{M}_5	2.4990	0.000531
6	M_{56e} 2.7906	0.001987	M_{67b} 2.8973	\bar{M}_6 2.8440	0.000907	\hat{M}_6	2.8597	0.000479

N.B.— \bar{M}_3 is the mean of \bar{M}_{23e} and \bar{M}_{34b} and so on. The symbol is introduced here as alternative to \hat{M} to simplify presentation.

TABLE 9
A. TOTAL DRY WEIGHT (ABSOLUTE VALUES)

Experimental Values			Estimated Values		
	Mean	Variance		Mean	Variance
W_{23e}	0.5588	0.0073	W_{23e} referred to \bar{M}_3	0.5558	0.0038
W_{34e}	1.8964	0.0609	W_{34e} referred to \bar{M}_3	1.9059	0.0317
			\bar{M}_4	2.2103	0.0323
W_{45e}	7.1056	0.5262	W_{45e} referred to \bar{M}_4	6.4716	0.2966
			\bar{M}_5	6.1605	0.2778
W_{56e}	11.4269	1.4780	W_{56e} referred to \bar{M}_5	13.4116	0.7578
			\bar{M}_6	12.8779	0.7695
W_{67e}	71.5562	4.8807	W_{67e} referred to \bar{M}_6	70.1886	2.4606

B. TOTAL DRY WEIGHT (LOGARITHMIC VALUES)

W_{23e}	1.6433	0.007795	W_{23e} referred to \bar{M}_3	1.6529	0.004060
W_{34e}	0.2128	0.004226	W_{34e} referred to \bar{M}_3	0.2044	0.002190
			\bar{M}_4	0.2777	0.002244
W_{45e}	0.8144	0.002314	W_{45e} referred to \bar{M}_4	0.7644	0.001272
			\bar{M}_5	0.7424	0.001232
W_{56e}	1.0184	0.002451	W_{56e} referred to \bar{M}_5	1.0868	0.001263
			\bar{M}_6	1.0760	0.001304
W_{67e}	1.8522	0.000191	W_{67e} referred to \bar{M}_6	1.8424	0.000092
			W_{23e} referred to \hat{M}_3	1.7062	0.001542
			W_{34e} referred to \hat{M}_3	0.2517	0.001146
			\hat{M}_4	0.2542	0.001160
			W_{45e} referred to \hat{M}_4	0.7462	0.000679
			\hat{M}_5	0.7473	0.000658
			W_{56e} referred to \hat{M}_5	1.0915	0.000527
			\hat{M}_6	1.0930	0.000547
			W_{67e} referred to \hat{M}_6	1.8470	0.000067

C. TOTAL DRY WEIGHT (ANTILOGARITHMIC VALUES)

W_{23e}	0.4398	0.0080	W_{23e} referred to \bar{M}_3	0.4497	0.0043
W_{34e}	1.6323	0.0597	W_{34e} referred to \bar{M}_3	1.6010	0.0298
			\bar{M}_4	1.8954	0.0427
W_{45e}	6.5223	0.5219	W_{45e} referred to \bar{M}_4	5.8130	0.2278
			\bar{M}_5	5.5259	0.1995
W_{56e}	10.4328	1.4144	W_{56e} referred to \bar{M}_5	12.2124	0.9987
			\bar{M}_6	11.9124	0.9811
W_{67e}	71.1541	5.1270	W_{67e} referred to \bar{M}_6	69.5665	2.3606
			W_{23e} referred to \hat{M}_3	0.5084	0.0021
			W_{34e} referred to \hat{M}_3	1.7852	0.0194
			\hat{M}_4	1.7956	0.0198
			W_{45e} referred to \hat{M}_4	5.5744	0.1119
			\hat{M}_5	5.5886	0.1090
			W_{56e} referred to \hat{M}_5	12.3452	0.4258
			\hat{M}_6	12.3880	0.4451
			W_{67e} referred to \hat{M}_6	70.3072	1.7559

The contrast of absolute estimates and the antilogarithms of the logarithmic estimates is essentially parallel to a contrast of arithmetic and geometric means, the latter always being the smaller for distributions of positive values.

(e) *Improved Estimates of R , E_{LW} , and E_{LA}*

Direct substitution of the values of \bar{W} , \bar{LW} , \bar{LA} , and \hat{W} , \hat{LW} , \hat{LA} in the formulae for R , E_{LW} , and E_{LA} and their standard errors, gives the values of Table 6.

Generally speaking the gains in precision in the relative growth rate and net assimilation rates through the use of ratings are quite substantial, principally owing to the fact that the variance of the estimated mean rating is less important since its coefficient involves the differences of regression coefficients or weighted regression coefficients. This also means that there is little advantage in the use of maximum likelihood estimates in place of mean rating at first harvest except where the regression coefficients on the logarithmic basis for W , LW , or LA for the two harvests are very different.

The gains in precision using ratings are indirectly confirmed in this experiment by the much more regular changes in R etc., from one interval to the next in comparison of treatments, R being adjusted independently of course for each treatment.

The principal use of $R(E_{LW}, E_{LA})$ lies in the comparison of treatments over the same period of time and the variance of differences in R between treatments will be the sum of the variances of R for the separate treatments. An unbiased estimate of the variance of the difference in R of the same treatment but not consecutive intervals is given by the sum of the separate variances for estimates based on mean rating at harvest. For estimates based on maximum likelihood rating the sum of variances would require a correction analogous to the correction for differences in \hat{W} in the previous section. For differences of R from consecutive intervals the sampling error in the material common to the two intervals will introduce positive corrections to the sum of the separate variances, whether ratings are used or not. For example, using natural logarithms of the weights, the variances of R (see 18) for two successive intervals, without ratings, can be expressed as

$$\frac{1}{(t_2 - t_1)^2} (\sigma^2 \bar{U}_{2e} + \sigma^2 \bar{U}_{1e}), \frac{1}{(t_3 - t_2)^2} (\sigma^2 \bar{U}_{3e} + \sigma^2 \bar{U}_{2e}).$$

The correction to the sum of these to give an unbiased estimate of the differences of R 's from the consecutive intervals is

$$\frac{2\sigma^2 \bar{U}_{2e}}{(t_2 - t_1)(t_3 - t_2)}.$$

TABLE 6
RELATIVE GROWTH RATE, R AND NET ASSIMILATION RATES, E_{LW} AND E_{LI}

Interval	Absolute				Logarithmic							
	Not Rated		Rated		Not Rated		Rated		Not Rated		Rated	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
Relative Growth Rate, R												
3-4	0.1746	0.0287	0.1760	0.0049	0.1757	0.0055	0.1873	0.0361	0.1814	0.0075	0.1794	0.0073
4-5	0.1887	0.0236	0.1535	0.0061	0.1579	0.0083	0.1979	0.0266	0.1601	0.0074	0.1618	0.0077
5-6	0.0679	0.0211	0.1107	0.0038	0.1112	0.0044	0.0671	0.0227	0.1133	0.0038	0.1132	0.0048
6-7	0.1310	0.0079	0.1211	0.0036	0.1199	0.0025	0.1423	0.0085	0.1261	0.0047	0.1240	0.0031
Net Assimilation Rate, E_{LW}												
3-4	0.2499	0.0411	0.2520	0.0084	0.2517	0.0081	0.2670	0.0516	0.2586	0.0105	0.2560	0.0106
4-5	0.2906	0.0375	0.2350	0.0121	0.2408	0.0130	0.3020	0.0422	0.2428	0.0117	0.2448	0.0116
5-6	0.1135	0.0354	0.1865	0.0072	0.1864	0.0072	0.1110	0.0376	0.1876	0.0077	0.1877	0.0077
6-7	0.3429	0.0209	0.3183	0.0121	0.3141	0.0083	0.3616	0.0228	0.3338	0.0147	0.3267	0.0101
Net Assimilation Rate, E_{LI}												
3-4	0.2322	0.0396	0.2342	0.0085	0.2346	0.0083	0.2402	0.0465	0.2325	0.0093	0.2333	0.0095
4-5	0.3086	0.0391	0.2504	0.0132	0.2568	0.0140	0.3190	0.0436	0.2557	0.0116	0.2570	0.0116
5-6	0.1189	0.0364	0.1957	0.0052	0.1957	0.0054	0.1161	0.0395	0.1979	0.0060	0.1980	0.0061

Logarithmic estimates of R , E_{LW} , and E_{LA} tend to be higher than the absolute values in this data while standard errors are of the same order. These relations hold also for other treatments.

VII. GENERAL DISCUSSION

In attempting to assess the value of the technique, one must not overlook the computational effort that is involved. There will be obvious cases where random sampling of relatively large numbers of plants (e.g. harvests 1 and 2 of the example) can be accomplished with ease in the time available for harvesting and preparation of the material. In such cases there would be little point in adopting the procedure.

There are also types of experiment, particularly with potted plants, where it is possible to gain statistical control of plant variability by allotting all treatments at random within size groups based on leaf area prior to application of treatments. Any additional gain in precision resulting from the rating technique would rarely justify the computational effort involved unless there was evidence of interaction in the logarithms of yields between treatments and size classes which would be associated with different regression slopes of yield on size for different treatments.

For this event the weights would be referred back to the general mean of the pretreatment ratings and the rating values would be regarded as fixed for repeated sampling. The variance expressions given would then be modified by the omission of reference point variation and the actual deviates of the particular mean ratings from the general mean would be used in conjunction with errors in regression coefficients instead of the expectation of these deviations. Thus, under these conditions (8) would become

$$\frac{\sigma^2_{y_{2e}, x_{2b}}}{n_2} + \frac{\sigma^2_{y_{1e}, x_{1e}}}{n_1} + \sigma^2_{b_2}(\bar{x}_{2b} - \hat{x})^2 + \sigma^2_{b_1}(\bar{x}_{1e} - \hat{x})^2$$

and this would simplify further on the assumption of the same array variance and regression slope for the two particular distributions to

$$\sigma^2_{y,x} \left(\frac{1}{n_2} + \frac{1}{n_1} \right) + \sigma^2_b(\bar{x}_{2b} - \bar{x}_{1e})^2.$$

In a similar manner the expressions for R , E_{LW} , and E_{LA} would be modified and in general simplified.

The technique outlined in this paper has its chief value in cases where the treatments are operative from germination or from a stage where ratings are unavailable or of little use and where the amount of material at each harvest is severely restricted, for example because of inherent difficulties in harvesting within some suitable unit of time. In growth experiments it is not uncommon for plants to double or even to treble their size each week during early stages of growth, so that the size of the plants soon sets the limit to the number that can be handled in one day. Then, too, the need for precision is

perhaps even greater in growth experiments than in yield trials, for the interest centres more in weight increment than in final weight. It is this gain in precision which is so important for experiments on field-grown crops, where plant variation tends to be very great.

Other worthwhile applications of the technique are likely to be in connection with individual leaf studies where high precision is desirable with limited material, and to studies of fruit growth. An experiment has come to our notice in which it was desired to compare fresh and dry weight increments of fruit from trees which had been subjected to varying degrees of thinning. The removal of the necessarily large successive samples of fruit was such as to vitiate the treatments under comparison, and it seems probable that the use of ratings based on an estimate of fruit volume (e.g. the cube of mean diameter) might reduce the necessary sample size to such an extent that treatment would not be seriously affected. That fruit volume is likely to be a good basis for rating in such studies is indicated by the data of Ross (1946) for tomato fruits.

A point which cannot be too strongly emphasized is that the rating must be highly correlated with the yield function for best results. Leaf area is probably the best basis for rating in young plants, but stem measurements (e.g. height \times girth) might be better for mature plants. If photographic standards are used for leaf-area rating, it is highly desirable that the same person should make all the estimates.

In the example of this paper a comparison has been made of the use of the absolute and the logarithmic data. In applying the analysis of variance in the statistical treatment of growth data, it has usually been found necessary to use the logarithmic transformation in order to eliminate the correlation of class means with their standard errors. Furthermore, it was known (Goodall 1945) that the logarithmic data were likely to give approximately parallel regression lines (see also Figs. 3, 4, and 5 of this paper). However, the gain in information using ratings seems to be as great with the untransformed data, so there seems little point in using the transformation, especially as the actual measurements were found to be slightly more compatible with the assumptions underlying the development of the theory.

The computational effort concerned with the determination of the maximum likelihood estimates of the mean ratings could be eliminated in some types of experiment. Thus, if the time interval between the first and the last of the harvests is short, the weight-rating correlations will all be high, and it may be possible to rate all the plants of the experiment at one time. In such a case the mean rating of each treatment automatically becomes the maximum likelihood estimate for the treatment. Even if the weight-rating correlation is not maintained, or it is not possible to rate all the plants at once, the same principle could be extended to two or more groups of harvests. By ignoring the rating linkages between such groups, one would lose a little information, but this is likely to be small in comparison with the computational labour of recovering this information.

VIII. ACKNOWLEDGMENT

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INVESTIGATIONS ON THE STABILITY AND DETERMINATION OF DEHYDROASCORBIC ACID

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Summary

The time intervals for complete reduction of dehydroascorbic acid by hydrogen sulphide at pH 4, 5, 6, and 7 at temperatures of 0°, 25°, and 40°C. have been determined.

The times are given for half destruction of dehydroascorbic acid at pH 0, 1, 2.2, 3, 4, 5, 6, and 7 and at temperatures of 0°, 25°, 40°, 70°, and 100°C. The rate of destruction is least at pH 2.2. Borate considerably accelerates the destruction of dehydroascorbic acid, particularly at higher pH.

A procedure is described which increases the specificity of the determination of dehydroascorbic acid by indophenol titration. This involves extraction at pH 2, reduction with hydrogen sulphide at pH 5.3, and destruction of dehydroascorbic acid with borate to obtain a blank. This blank is considerable in extracts from a number of fruit and vegetable products.

Little dehydroascorbic acid was found in most of the fresh fruits and vegetables and in the processed juices examined, but appreciable concentrations were found in the fresh juices.

I. INTRODUCTION

This paper is concerned with the determination of the stability of dehydroascorbic acid over a wide range of temperature and pH. Such data can be used for predicting the retention of dehydroascorbic acid by various processed foods, and can also be applied in the determination of dehydroascorbic acid by reduction with hydrogen sulphide (to ascorbic acid) and subsequent indophenol titration. It is desirable to extract at the pH of maximum stability and to obtain a blank titre after destruction of the dehydroascorbic acid under suitable conditions.

Penney and Zilva (1943) found that the rate of conversion of dehydroascorbic acid to 2, 3-diketogulonic acid in citrate-phosphate buffers at 38°C. increased with pH over the range 4.0-7.4. In a more acid range, the rate was found to increase with increasing concentration of hydrochloric acid from 0.1N to 1.0N at 25°C. In these experiments the loss of dehydroascorbic acid was followed both directly and by determination of the diketogulonic acid formed. The two methods gave very similar results except at pH 7.4, where there was evidence of further change. Borate buffer at pH 7.4 was found to stabilize the diketogulonic acid and give results which were in substantial agreement for the two methods. Roe and others (1948) found maximum stability of dehydroascorbic acid at pH 2-3.

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II. REDUCTION OF DEHYDROASCORBIC ACID

Before proceeding with the main investigation, it was necessary to determine the conditions for complete reduction of dehydroascorbic acid by hydrogen sulphide. The solutions of dehydroascorbic acid were obtained by oxidizing ascorbic acid (20 mg./100 ml.) in citrate-phosphate buffer with an exact equivalent of iodine. After bubbling hydrogen sulphide through the solution for fifteen minutes, it was allowed to stand in a stoppered flask. At intervals aliquots were pipetted into metaphosphoric acid solution (to reduce the pH to about 1). The hydrogen sulphide was then removed by passing carbon dioxide through the solution for fifteen minutes, and the reduced ascorbic acid was determined by titration. The minimum periods after saturation with hydrogen sulphide (by bubbling for fifteen minutes) necessary under these conditions for complete reduction are given in Table 1.

TABLE 1
MINIMUM TIME OF STANDING FOR COMPLETE REDUCTION
OF DEHYDROASCORBIC ACID WITH HYDROGEN SULPHIDE

pH	Minimum Time (min.) at		
	0°C.	25°C.	40°C.
4	90	15	0
5	45	5	0
6	30	0	0
7	15	0	0

The rates of reduction were not significantly affected by adding boric acid to the buffers to give a final concentration of 0.1M. The importance of boric acid will be shown in subsequent sections.

III. STABILITY OF DEHYDROASCORBIC ACID

(a) Effect of Temperature and pH

The destruction of dehydroascorbic acid was estimated at pH levels from 0.0 to 7.0 and at temperatures from 0° to 100°C. The solutions of dehydroascorbic acid were obtained by mixing 40 ml. of the appropriate buffer, 2 ml. of 0.5 per cent. ascorbic acid, and the exact equivalent of 0.01N iodine. In the experiments at 0°, 25°, and 40°C. the solutions were held at each temperature and 5 ml. aliquots were withdrawn at intervals. The data at 70° and 100°C. were obtained by keeping the bulk solutions at a low temperature, and holding each aliquot for the required period at the higher temperature. Each aliquot was reduced with hydrogen sulphide as described in the previous section and then titrated with 2,6-dichlorophenolindophenol (aliquots which were more acid than pH 4 were brought to pH 4 by addition of sodium acetate or disodium phosphate before reduction).

The precise investigation of the kinetics is complicated by the fact that the end products when treated with hydrogen sulphide give rise to some indo-

phenol-reducing material, but it is probable that the course of destruction of dehydroascorbic acid approximates a first order reaction. The results of Penney and Zilva (1943), confirmed by the author, show that diketogulonic acid gives about one-tenth the titre of the equivalent amount of dehydroascorbic acid. At pH 7, further changes result in the production of reducing material; after 30 minutes at 40°C. a solution of this pH developed, without any hydrogen sulphide treatment, a reducing titre equivalent to 7 per cent. of that of the original solution after reduction.

The results can be most usefully expressed as the time required for destruction of half the original dehydroascorbic acid. The titre after reduction should be approximately 55 per cent. of the original (50 per cent. due to dehydroascorbic acid and 5 per cent. due to diketogulonic acid). The times of half destruction, reported in Table 2, were obtained in 1.0N hydrochloric acid (pH 0), 0.25M oxalic acid (pH 1), and McIlvaine's citrate-phosphate buffers (pH 2.2-7). Results obtained in 1.4N sulphuric acid (pH 0), 0.1N hydrochloric acid (pH 1), 0.1M sodium acetate adjusted with sulphuric acid (pH 1-2), phthalate buffers (pH 2.2-6), and phosphate buffers (pH 6-7) did not differ by more than 10 per cent.

TABLE 2
TIMES OF HALF DESTRUCTION OF DEHYDROASCORBIC ACID

pH	Time of Half Destruction at				
	0°C.	25°C.	40°C.	70°C.	100°C.
0	24 hr.	2 hr.	30 min.	6 min.	1½ min.
1	5½ days	12 hr.	3 hr.	30 min.	7 min.
2.2	15 days	35 hr.	9 hr.	80 min.	17 min.
3	14 days	34 hr.	8 hr.	50 min.	8 min.
4	12 days	24 hr.	6 hr.	30 min.	3 min.
5	9 days	15 hr.	3 hr.	8 min.	1 min.
6	5 days	4½ hr.	35 min.	2 min.	< 1 min.
7	24 hr.	45 min.	6 min.	< 1 min.	< 1 min.

At all temperatures, dehydroascorbic acid has maximum stability at approximately pH 2. The stability decreases at both higher and lower pH levels. With increasing pH, the temperature coefficient of the reaction increases. From 0° to 40°C., the Q_{10} is approximately 2.5 at pH 0-4, 3.0 at pH 5, and 4.0 at pH 6-7.

The stability of dehydroascorbic acid is shown to vary greatly with both temperature and pH. The stability at 0°C. suggests the possibility of appreciable retention in many cold stored foods, particularly frozen foods. With regard to canned foods, the heat treatments used for sterilization would usually destroy most of the dehydroascorbic acid. Exceptions are most likely to be found in acid foods, particularly canned fruit juices. In such products, which usually have a pH of 2.5-4.0, dehydroascorbic acid is comparatively stable and only a short heat treatment is required for sterilization.

The destruction of dehydroascorbic acid does not involve atmospheric oxidation, and the rate of destruction was not significantly affected by bubbling either air or nitrogen through the solution. The addition of ten parts per million of copper (as copper sulphate), which catalyses the atmospheric oxidation of ascorbic acid, had no significant effect.

(b) *Effect of Borate*

Penney and Zilva (1943) have shown that diketogulonic acid is rendered more stable at pH 7.4 by the presence of borate, and the formation of reducing substances on treatment with hydrogen sulphide is thus eliminated. The possibility of using borate in the determination of dehydroascorbic acid was considered, as it would enable dehydroascorbic acid to be removed without giving rise to a residual titre.

Borate was found to accelerate the destruction of dehydroascorbic acid, an effect which was not apparent from the data of Penney and Zilva (1943), as destruction at pH 7.4 is extremely rapid whether in the presence or absence of borate.

The effect of borate was investigated at pH 0-7 by adding boric acid to the appropriate buffers to give a final concentration of 0.1M. As the borate prevented interference by diketogulonic acid, the time taken to reach half the original titre can be regarded as that necessary for half destruction of dehydroascorbic acid. The results are given in Table 3.

TABLE 3
EFFECT OF BORATE ON DESTRUCTION OF DEHYDROASCORBIC ACID

pH	Temp. (°C.)	Time of Half Destruction	
		Control	Borate
0	40	30 min.	30 min.
1	40	3 hr.	3 hr.
2.2	40	9 hr.	4 hr.
3	40	8 hr.	2 hr.
4	40	6 hr.	40 min.
	0	9 days	3½ hr.
5	25	15 hr.	30 min.
	40	3 hr.	11 min.
	0	5 days	60 min.
6	25	4½ hr.	7 min.
	40	35 min.	1½ min.
7	40	6 min.	1 min.

The accelerating effect of borate appears at approximately pH 2 and increases with increasing pH. The effect of boric acid both in accelerating the decomposition of dehydroascorbic acid and in stabilizing the diketogulonic acid formed is probably associated with its ability to react with hydroxyl groups.

IV. DETERMINATION OF DEHYDROASCORBIC ACID

(a) Methods of Obtaining Blank

Determination of dehydroascorbic acid in foods involves extraction, reduction with hydrogen sulphide, and titration with 2,6-dichlorophenolindophenol. The extracting solution should contain a stabilizer for ascorbic acid, e.g. oxalic acid, and should give an extract with a pH of approximately 2. This pH combines maximum stability of dehydroascorbic acid with satisfactory stability and extraction of ascorbic acid. It is desirable to increase the pH before reduction with hydrogen sulphide.

As substances other than dehydroascorbic acid may give indophenol-reducing material on treatment with hydrogen sulphide, it is essential to obtain a satisfactory blank. The blank is a measure of interfering substances and is obtained by destroying the dehydroascorbic acid before treatment of the solution with hydrogen sulphide and titration. The blank titre is subtracted from the total titre.

In preliminary investigations, a solution containing one per cent. of oxalic acid and 0.75 per cent. of sodium acetate was used for extraction. This gave an extract of approximately pH 2. The uncorrected figure (a) (see Table 4) was obtained by adding 2 ml. of 50 per cent. sodium acetate to a 10 ml. aliquot (to bring the pH to 5.3), reducing with hydrogen sulphide, and titrating. The pH of 5.3 combined adequate stability with sufficiently rapid reduction. Slight variations in the amount of sodium acetate added did not affect the pH appreciably.

Blank titres were obtained on 10 ml. aliquots after treating as follows:

(b₁) Add 2 ml. of 50 per cent. sodium acetate (to bring the pH to 5.3), allow to stand for 16 hours at 40°C. in an atmosphere of carbon dioxide, and subsequently reduce with hydrogen sulphide.

(b₂) Add 2 ml. of a solution containing 50 per cent. of sodium acetate and 3 per cent. of boric acid (which brings the pH to 5.3), allow to stand for one hour at 40°C. in a current of carbon dioxide, and reduce with hydrogen sulphide.

(b₃) Add 2 g. of Na₂HPO₄.12H₂O (to bring the pH to 7), allow to stand for one hour at 40°C. in a current of nitrogen, and reduce with hydrogen sulphide.

Carbon dioxide does not affect the pH in the first two procedures, but reduces the pH in the third procedure. Hence nitrogen was used instead.

Of the three methods for destroying dehydroascorbic acid, method (b₁) required an excessive time for destruction, but it was useful as a control for comparing the effect of borate. Method (b₂), which involved the use of borate, gave comparatively rapid destruction and a low residual titre. However, as there was a possibility of borate causing interference through combination with other substances, procedure (b₃), which gave as rapid destruction without the use of borate, was included. It was necessary to raise the pH to 7 to obtain rapid destruction in the absence of borate.

Solutions of dehydroascorbic acid and a number of possible interfering substances were analysed to obtain the uncorrected figure and each of the three blank figures. Each of the blanks was subtracted from the uncorrected figure to obtain a corrected figure for dehydroascorbic acid. All the results were calculated as mg. of ascorbic acid per 10 ml. aliquot.

The solutions contained in a 10 ml. aliquot 0.1, 0.2, 0.5, and 1.0 mg. of dehydroascorbic acid and the following amounts of possible interfering substances: 1.0 mg. of ascorbic acid; 0.2 mg. of tin (as $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$); 0.2 mg. of iron (as $\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$); 1.0 mg. of pyruvic acid; 1.0 mg. of 1,4-naphthoquinone; and 1.0 mg. of diketogulonic acid. The ferrous and stannous solutions had become partially oxidized before analysis. Both tin and iron may be found in canned foods. Derivatives of 1,4-naphthoquinone, such as vitamin K and juglone, are known to occur naturally. The diketogulonic acid was obtained by allowing dehydroascorbic acid to stand in 0.1N hydrochloric acid.

The results, which include original reducing material (without reduction by hydrogen sulphide), uncorrected figure after reduction, blanks, and corrected figures for dehydroascorbic acid, are given in Table 4.

TABLE 4
COMPARISON OF BLANKS AND CORRECTED FIGURES FOR DEHYDROASCORBIC ACID

Substance	Mg. in 10 ml. Aliquot	Original Reducing Material	Reducing Material after Reduction by Hydrogen Sulphide						
			Uncorrected Figure (a)	Blanks			Corrected Figure for Dehydroascorbic Acid		
				(b ₁)	(b ₂)	(b ₃)	(a-b ₁)	(a-b ₂)	(a-b ₃)
Dehydroascorbic acid	0.1*	0	0.109	0.023	0.011	0.018	0.086	0.098	0.091
„	0.2*	0	0.210	0.034	0.012	0.041	0.176	0.198	0.169
„	0.5*	0	0.514	0.051	0.014	0.062	0.463	0.500	0.452
„	1.0*	0	1.005	0.106	0.028	0.097	0.899	0.977	0.908
Ascorbic acid	1.0	1.000	1.000	0.990	1.000	0.941	0.010	0	0.059
Tin	0.2	0	0.012	0.012	0.012	0.012	0	0	0
Iron	0.2	0.120	0.314	0.314	0.314	0.233	0	0	0.081
Pyruvic acid	1.0	0	0.015	0.015	0.015	0.030	0	0	-0.015
1,4-Naphthoquinone	1.0	0.038	0.886	0.610	0.827	0.784	0.276	0.059	0.102
Diketogulonic acid	1.0	0	0.123	0.058	0.066	0.150	0.065	0.057	-0.027

* Expressed as ascorbic acid.

The best results were generally obtained with blank (b₂), in which borate was used for accelerating the destruction of dehydroascorbic acid. The figure obtained by subtracting this blank gave maximum recovery of dehydroascorbic acid (98-100 per cent.) and no interference by ascorbic acid, tin, iron, or pyruvic acid. Interference by 1,4-naphthoquinone was only 7 per cent. of the uncorrected figure and was considerably less than that obtained by subtracting

either of the other blanks. Interference by diketogulonic acid was reduced to about half the uncorrected figure.

It is of interest that the residual titre after destruction of dehydroascorbic acid in the presence of borate was much lower than that given by pre-formed diketogulonic acid in the presence of borate. This suggests that a borodiketogulonic acid complex was formed directly by the action of borate on dehydroascorbic acid.

The slight interference by ascorbic acid in (a-b₁) and the greater interference in (a-b₃) were probably due to oxidation of ascorbic acid. Any ascorbic acid oxidized during the period of standing naturally appeared as dehydroascorbic acid. It is difficult to avoid slight oxidation on standing for sixteen hours at pH 5.3. If the period of standing was reduced to one hour by the use of borate, oxidation was negligible. At pH 7 (blank b₃) oxidation of ascorbic acid was more rapid, and it was difficult to obtain nitrogen completely free from oxygen. The figure given in Table 4 was obtained by displacing the air above the solution with nitrogen purified by passing through alkaline pyrogallol. If the nitrogen was bubbled continuously, more rapid oxidation occurred.

Sulphur dioxide seriously interfered with all of the possible procedures for determining dehydroascorbic acid. Solutions of sulphur dioxide gave a variable titre after treatment with hydrogen sulphide, and holding at pH 5-7 increased this variability.

(b) Suggested Procedure

The following solutions are required:

- (i) 10 g. of oxalic acid, (COOH)₂·2H₂O, and 7.5 g. of sodium acetate, CH₃COONa·3H₂O, per litre.
- (ii) 100 g. of sodium acetate per 200 ml.
- (iii) 6 g. of boric acid, H₃BO₃, and 100 g. of sodium acetate per 200 ml.
- (iv) 5N sulphuric acid.
- (v) 0.2 g. of 2,6-dichlorophenolindophenol per litre (equivalent to approximately 0.1 mg. of ascorbic acid per ml.). Make up in phosphate buffer of pH 7.2 and standardize with ferrous ammonium sulphate.

Extract 20 g. of material with solution (i) and make up to 200 ml. with solution (i). Procedures (a) and (b₂) are to be carried out on 10 ml. aliquots in 50 ml. conical flasks.

(a) Add 2 ml. of solution (ii), and immediately bubble H₂S for 15 min. Stopper, and allow to stand for 30 min. Then add 2 ml. of 5N H₂SO₄ (which reduces the pH to 1.3), bubble CO₂ for 15 min., and titrate.

(b₂) Immerse 50 ml. conical flask containing 2 ml. of solution (iii) in bath at 40°C. for 5 min. Pipette 10 ml. of extract, and bubble CO₂ for one hour. Then bubble H₂S for 15 min. and continue as for (a).

Calculate concentration of dehydroascorbic acid from (a-b₂).

This procedure is suggested as one method of determining dehydroascorbic acid by indophenol titration. It is certainly more specific than simple reduction

and titration without use of a blank. Without extensive analyses, including biological assays, it is not possible to compare this method adequately with alternative procedures, such as those of Lugg (1942) and Roe and others (1948).

(c) *Estimation of Dehydroascorbic Acid in Various Products*

The suggested procedure was applied to a number of fresh and processed foods which were likely to contain dehydroascorbic acid. They included fresh apples, fresh and canned apple juice, fresh tomatoes, fresh and canned tomato juice, fresh and canned orange juice, fresh beetroot, and honey. The apples (Granny Smith variety) were semi-ripe. The canned juices had been stored for about twelve months.

Fresh beetroot and honey were included, as the presence of interfering substances has been suggested in these products. Beetroot extracts were difficult to titrate either visually or potentiometrically, and approximate titres were obtained by the chloroform method of McHenry and Graham (1935). The results, which include ascorbic acid by direct titration, are given in Table 5. The uncorrected figure for dehydroascorbic acid is obtained by subtracting ascorbic acid (obtained by direct titration) from (a).

TABLE 5
DETERMINATION OF DEHYDROASCORBIC ACID IN FRESH AND PROCESSED FOODS

Product	Ascorbic Acid (mg. per 100 g.)	Dehydroascorbic Acid (as mg. of Ascorbic Acid per 100 g.)			
		(a)	(b ₂)	Uncorrected Figure	Corrected Figure (a-b ₂)
Fresh apple (very small)	15.1	21.2	15.4	6.1	5.8
Fresh apple (medium)	8.6	13.2	9.9	4.6	3.3
Fresh apple juice (very small)	0	17.2	7.3	17.2	9.9
Fresh apple juice (medium)	0	8.6	5.5	8.6	3.1
Canned apple juice	0	3.2	2.4	3.2	0.8
Fresh tomato	20.2	22.0	21.1	1.8	0.9
Fresh tomato juice	12.1	17.7	14.5	5.6	3.2
Canned tomato juice	6.4	12.6	11.7	6.2	0.9
Fresh orange juice	37.5	41.7	40.7	4.2	1.0
Canned orange juice	51.7	54.1	54.1	2.4	0
Fresh beetroot	25.4	31.2	31.2	5.8	0
Honey	1.5	5.1	8.0	3.6	—

In all products the corrected figure for dehydroascorbic acid was less than the uncorrected figure. Interference was considerably reduced by the use of a blank. Honey gave somewhat anomalous results in which (b₂) was greater than (a).

On examining the corrected figures, it is seen that the immature fresh apples contained an appreciable concentration of dehydroascorbic acid. The larger apples, which were still comparatively immature, had less dehydroascorbic acid, and the concentration in fresh tomatoes and beetroot was negligible. As expected, the fresh juices all contained significant levels of dehydroascorbic acid resulting from oxidation during extraction. The concentration in orange juice was, however, slight, as oxidation of ascorbic acid proceeds slowly in this product. Practically complete oxidation occurred during the extraction of apple juices.

In the canned juices, the concentration of dehydroascorbic acid was slight in tomato, and negligible in apple and orange. The heat sterilization used in canning, combined with the long storage period, was sufficient to destroy practically all the dehydroascorbic acid.

V. ACKNOWLEDGMENTS

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SEASONAL VARIATIONS IN THE VITAMIN A CONTENT OF VICTORIAN BUTTERFAT

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Summary

The total vitamin A potency of butterfat (derived from cheese or butter) has been determined on samples from three different Victorian districts and from King Island in Bass Strait in 1945 and 1947-48. Carotene was determined colorimetrically and the Carr-Price test was used for vitamin A. The glycerol dichlorhydrin reagent was too insensitive.

Sufficient samples were obtained from west Gippsland and the Western District (Victoria) to show the seasonal fluctuation over a whole year. There is a minimum value for total vitamin A potency in the late summer or early autumn when the pastures have dried off and a maximum in the late winter or early spring following the appearance of new growth.

Values obtained in 1945 were higher than those in the 1947-48 series. It is suggested that this is a concentration effect in that in 1945 normal quantities of carotene were ingested by the cows and secreted in a much smaller volume of milk than in the same period in 1947. It is apparent that there can be wide variations between values obtained each year at the same time.

Comparison of samples of February (1949) butterfats from the areas studied, with one from Orbost in east Gippsland where pastures remain green and plentiful in late summer, underlined the importance of carotene in the feed and suggested that seasonal fluctuations in this area would be very much smaller than in the rest of Victoria.

Comparison with figures in the literature shows that Victorian butterfats compare favourably with those in other parts of the world so far as vitamin A potency is concerned, the normal values probably being of the order of 18,000 I.U./lb. Minimum values of the order of 11,000 I.U./lb. are likely in late summer.

I. INTRODUCTION

It has long been recognized that the vitamin A content of butterfat is subject to seasonal fluctuations which are dependent largely on the variations in the feed available to the cow (Price 1931; Fraps and Treichler 1932; Gillam *et al.* 1933; Converse, Wiseman, and Meigs 1934; Jenness and Palmer 1945; Kon 1945; Parrish *et al.* 1946). Thus in the northern parts of the United States and in Europe, winter stall feeding of cattle with low carotene feeds is linked with substantial decreases in vitamin A potency in the butterfat (Morgan and Pritchard 1937; Dornbush, Peterson, and Olson 1940; Hvidsten 1943; Lord 1945). At the same time, Kunerth and Riddell (1938) have shown that cattle living under drought conditions produced butterfat with a very much reduced vitamin A potency, the carotene content being particularly low.

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It seemed likely, therefore, that a study of seasonal fluctuations of vitamin A potency of Australian butterfat which is obtained from cows which are at pasture the year round would show minima in the late summer when the pastures, having dried off, would be low in carotene. Furthermore, it was reasonable to expect that the overall average values for vitamin A potency would be higher than those obtained in countries with greater seasonal differences.

The work reported here was begun in 1945, but had to be laid aside for a time because of circumstances beyond the control of the authors. In the meantime, Parrish *et al.* (1946) reported the deleterious effect of hot summer weather on vitamin A values of Kansas butter and Barnicoat (1947) showed conclusively that (for the season 1935-36) the minimum values for vitamin A potency in New Zealand (North I.) butter were, in fact, found in late summer (February) at the time when the pasture normally tends to dry up.

It would appear that the results reported in this paper are the first referring to Australian butterfat and that the conditions existing, in Victoria at least, closely parallel those obtaining in the North Island of New Zealand.

II. EXPERIMENTAL

(a) *The Determination of Vitamin A*

The Carr-Price (1926) method was used. Carotene also reacts with antimony trichloride to give a blue colour, but the rates of reaction are quite different according to Oser, Melnick, and Pader (1943), as carotene does not give its maximum blue colour until some two hours have elapsed. The error introduced by reading the colour formed by a mixture of vitamin A and carotene in the first four seconds after addition of the antimony trichloride, and converting this to units of preformed vitamin A is extremely small in such products as butter where the carotene contributes only a small proportion of the total vitamin A potency. This error has been ignored in this work.

(b) *The Determination of Carotene*

In butterfat, by far the greater part of the yellow colour normally encountered is β -carotene and some workers, e.g. Booth *et al.* (1933), ignore the small amount of xanthophyll and accept the intensity of the yellow pigmentation as a measure of the carotene present. That this is virtually correct has been demonstrated by Gillam (1934) who showed that the ratio of carotene to xanthophyll in English butter was 14:1 by weight. This is so constant that one is quite justified in accepting 94 per cent. of the absorption at 455-460 m μ as a measure of the carotene present.

In the early part of this work (1945) the determinations were carried out according to the method of Fraps, Kemmerer, and Greenberg (1940) in which the xanthophyll was removed from the solution of the unsaponifiable fraction in petroleum ether by shaking with "light MgCO_3 " before the solution was

placed in the colorimeter. The results so confirmed those of Gillam that in the later studies 94 per cent. of the total was accepted as carotene.

(c) Reagents

Chloroform.—Pure chloroform was washed two or three times with water, dried over Na_2SO_4 , refluxed and finally distilled. It was stored in a dark brown bottle away from the light.

Carr-Price Reagent.—Antimony trichloride (C.P.) was distilled directly from a retort into chloroform (250 ml.) until 75 g. had been added. The solution was set aside to come to saturation equilibrium in the presence of excess crystalline SbCl_3 . It was stored in the dark at 20°C .

Peroxide-free Ether.—Ethyl ether (B.P.) was shaken with a saturated solution of ferrous sulphate, separated, dried over Na_2SO_4 and distilled in an all glass still painted black on the outside.

Alcohol.—Absolute alcohol (C.P.) was distilled once.

Petroleum Ether.—The fraction of an industrial solvent (Shell X222) boiling below 80°C . was distilled and dried over Na_2SO_4 .

Magnesium Carbonate.—Light, B.D.H.

Acetic Anhydride.—C.P.

Potassium Hydroxide 60%.—This was prepared from the C.P. reagent and distilled water.

(d) Preparation of Samples

Four dairying districts have been studied. They are Allansford-Garvoc in the Western District of Victoria, Drouin in Gippsland, Leitchville in the Murray Valley irrigation area, and King Island in Bass Strait. Both butter and cheese have been used as the source of fat, the latter when it was inconvenient or impossible to obtain butter from the factories under consideration. It was thought safe to do this as the work of Dearden *et al.* (1946) points to the complete conservation of vitamin A /g. of fat during the manufacture and maturing of cheese and the unpublished work of one of the authors (W.M.B.) has shown that there is no loss of vitamin A potency even in the processing of cheese.

Samples were drawn at random from production, an 8 oz. pat, if butter, and for cheese, production samples covering 2-3 days. The butter samples were melted and filtered through a coarse, dry filter paper which removed water and curd. Cheese samples were taken with the conventional cheese trier, minced, and held at $50-60^\circ\text{C}$. for 1-2 hours, whereupon the fat separated. It, too, was decanted through a coarse, dry filter paper.

(e) Saponification

Twenty g. of the filtered fat was weighed into a 250 ml. conical flask painted black on the outside and saponified on a hot plate with 12.5 ml. of 60 per cent. KOH and 25 ml. of absolute alcohol. One hundred ml. of cold water was then added and the flask cooled under running water. The solution

was then extracted first with 100 ml. and then with three lots of 50 ml. of peroxide-free ether. The ethereal extracts were combined, washed with four 50 ml. lots of water, dried overnight over anhydrous sodium sulphate, and then adjusted to some convenient dilution and divided into two parts. The ether was boiled off each part and the flasks blown out with nitrogen gas. The dry unsaponifiable matter left in the flasks was dissolved, in one case in 25 ml. of dry chloroform, and in the other, in 25 ml. of petroleum ether. At all stages the solutions were shielded from strong light.

(f) *Determination of Vitamin A and Carotene with the Lange Photo-Electric Colorimeter (1945)*

The Lange photo-electric colorimeter consists of two selenium photo-electric cells activated by a common light source and balanced by suitable adjustments of iris diaphragms and resistances. It is graduated to read directly in extinction or percentage absorption. For the Carr-Price test, a yellow Wratten filter was used and the instrument was standardized from day to day with a copper sulphate solution (34.64 g. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ /500 ml.).

A diluted fish liver oil kindly supplied by Nicholas Pty. Ltd. was used as a standard. It contained 3025 I.U. of vitamin A/g. The tubes containing the chloroform solutions of unsaponifiable matter were placed in the instrument and 9 ml. of Carr-Price reagent added rapidly from an automatic pipette. The speed of addition was sufficient to mix the solution and the galvanometer deflection was read at the point of temporary stability. From the results obtained a standard curve was constructed.

In assaying unknown samples, 20 g. of butterfat was saponified and 1 ml. of the final chloroform solution of the unsaponifiable matter (equivalent to 0.4 g. of fat) was taken for the final determination. The vitamin A present was read off from the standard curve.

Carotene was determined colorimetrically using the deep blue filter supplied with the instrument, petroleum ether to set the zero point, and a solution of potassium dichromate (0.018 g./100 ml.) to standardize the instrument.

A standard curve was obtained by diluting with petroleum ether a sample of peanut oil containing 0.0364 g. of pure β -carotene/250 ml. and plotting deflection against $\mu\text{g.}$ of β -carotene.

The butterfat samples were saponified and the unsaponifiable material dissolved in petroleum ether (100 ml.) which was then shaken with 5 g. of "light magnesium carbonate" to remove xanthophyll. After centrifuging the solution was read in the colorimeter and the β -carotene present determined by reference to the standard curve.

(g) *Determination of Vitamin A and Carotene with the Spekker Absorptiometer*

It has already been pointed out by Innes and Birch (1945) that the Spekker absorptiometer used normally as a null point instrument is not satisfactory for the measurement of the evanescent antimony trichloride colour.

However, these authors describe a modification of the use of this instrument by which it is possible to obtain reliable measurements of the Carr-Price colour. This method has been followed with further slight modification to permit the use of the galvanometer actually supplied with the absorptiometer.

A diluted fish liver oil, standardized by the Carr-Price procedure on the whole oil, was made available by Nicholas Pty. Ltd. It contained 2,000 I.U. of vitamin A/g. and was used to prepare a standard curve. For each determination, 6 ml. of antimony trichloride solution and 2 drops of acetic anhydride (to prevent any precipitation of antimony oxychloride) were placed in the cell. With this in place the setting of the instrument was checked and 1 ml. of vitamin A solution rapidly blown in from a wide bore pipette. The mercury switch button was kept depressed and the maximum rest point of the galvanometer noted.

In assaying unknown samples, 1 ml. of the chloroform solution of the unsaponifiable matter was used and the vitamin A content calculated from the graph.

The determination of carotene is a normal colorimetric procedure and a graph was constructed for β -carotene (the pure crystals) in concentrations of from 0.64 to 16 $\mu\text{g./ml.}$ of petroleum ether against Spekker drum readings using the violet filters supplied with the instrument, and a cell containing water as the blank. Unknown samples were assayed by comparing the drum reading of the petroleum ether solution of the unsaponifiable matter with the standard graph. As already indicated, 94 per cent. of this value represents the β -carotene content.

(h) The G.D.H. Reagent for Vitamin A Determination

The obvious disadvantages of the Carr-Price reagent have led to a search for another reagent. One of the latest suggestions is that of Sobel and Werbin (1945, 1946, 1947) who used the reaction of glycerol dichlorhydrin (1,3-dichloro-2-hydroxypropane), referred to as G.D.H., with vitamin A to measure the potency of the latter. Several attempts were made to use this reagent but all failed as it was far too insensitive even when "activated" by distillation over SbCl_3 .

III. RESULTS AND DISCUSSION

Vitamin A values were determined directly as International Units by comparison with an oil so standardized. Carotene was determined as $\mu\text{g.}$ of β -carotene and was converted to vitamin A potency on the basis of 1 I.U. = 0.6 $\mu\text{g.}$ The results are concerned only with butterfat from bulk milk as supplied to dairy factories and no account could be taken of variations due to breed differences.

Results obtained from Gippsland and Western District areas are shown in the figures (Figs. 1, 2, 3) where total vitamin A and carotene potencies are graphed with time. The curve for the year 1947-48 shows a definite seasonal fluctuation in total vitamin A potency with a minimum in the late summer or

early autumn when the pastures in all three districts have dried off, and a peak in the spring following the appearance of new lush growth.

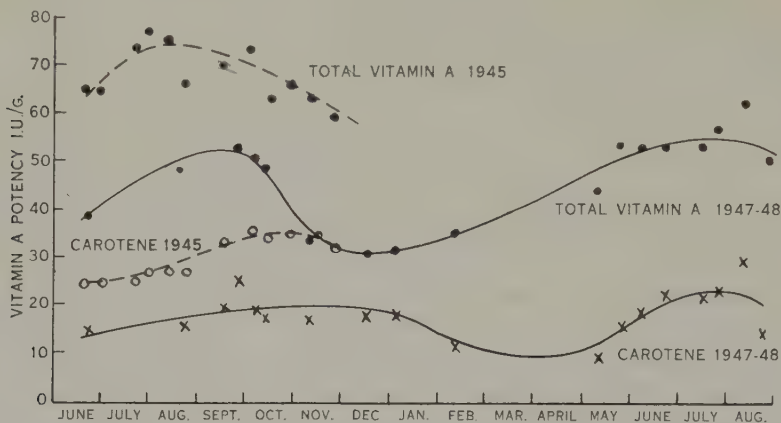


Fig. 1.—Seasonal fluctuations in vitamin A and carotene at Drouin (Gippsland).

As the Allansford and Garvoc factories are only some thirteen miles apart and draw milk from virtually the same area, a much smaller number of samples was taken from the latter factory which was used merely to confirm the trend shown by the Allansford results.

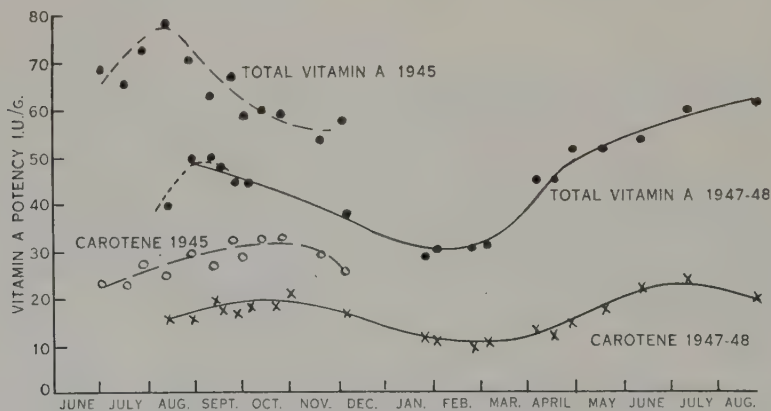


Fig. 2.—Seasonal fluctuations in vitamin A and carotene at Allansford (Western District).

Values for Leitchville and King Island are shown in Figures 4 and 5 respectively. It is unfortunate that these two areas could not be studied for a whole year, as one would expect to find smaller fluctuations in these districts than in the others: Leitchville is an irrigation district and King Island has abundant rain and cool summer temperatures. February butters from these areas are compared with Gippsland and Western District butterfat later (see Table 4).

A statistical analysis* has been made of the effect of rainfall on vitamin A and carotene in butterfat from Allansford and Drouin where complete yearly cycles were available. The total rainfall for the fortnight before the date of

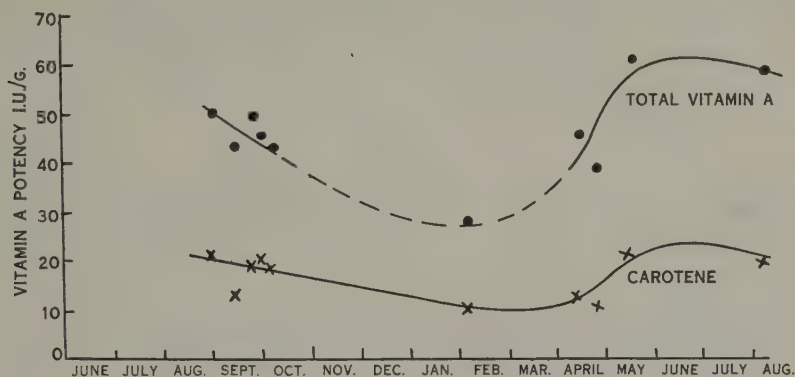


Fig. 3.—Seasonal fluctuations in vitamin A and carotene in butterfat at Garvoc (Western District), 1947-48.

test and for the fortnight before that were worked out and correlated with these two factors. For both Allansford and Drouin the effect on vitamin A was very much more pronounced than that on carotene. Moreover, it was found that the effect was much greater at Allansford than at Drouin. For the Allansford data the regression of vitamin A on the total rainfall for the preceding fortnight was significant at the 1 per cent. level, and that of carotene at the 5 per cent. level.

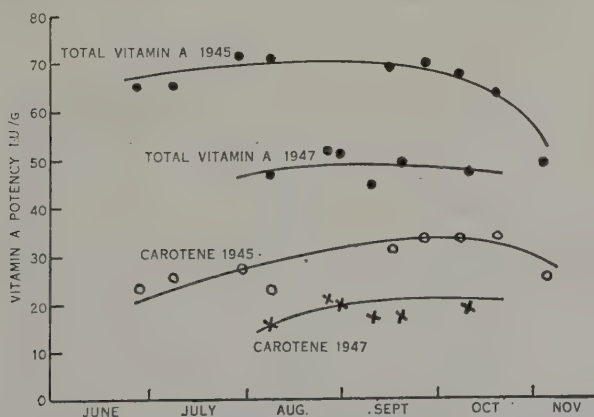


Fig. 4.—Seasonal fluctuations in vitamin A and carotene at Leitchville (northern irrigation area).

At Drouin neither of the regressions was significant. In no case was there any significant effect of the rainfall between 4 weeks and 2 weeks prior to test. This may be due to the greater total rainfall in this district which

* The authors are indebted to Mr. E. J. Williams, Section of Mathematical Statistics, C.S.I.R.O., for this treatment of their results.

averages about 34 per cent. more per annum than Allansford. While the relationships found are not very consistent or very impressive it may be taken as established that rainfall, through its effect on the chemical composition of the grass, does affect the vitamin A and carotene content of the butter produced. There are, of course, other factors such as temperature and soil which are concerned with pasture production.

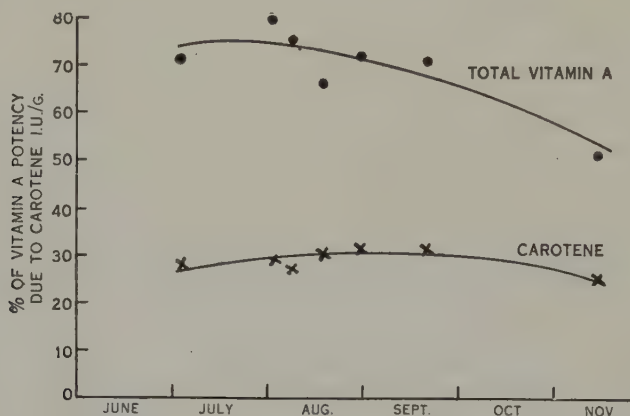


Fig. 5.—Seasonal fluctuations in vitamin A and carotene on King I. (Bass Strait), 1945.

From the vitamin A aspect the year may be subdivided into three periods, August-December, January-March, and April-July. The average vitamin A potency/g. of butterfat for all assays done in these periods is shown in Table 1.

TABLE 1
AVERAGE VITAMIN A POTENCY I.U./G. OF BUTTERFAT

District	Year	Aug.-Dec.	Jan.-Mar.	Apr.-July
Drouin	1945	68.0		
Drouin	1947-48	46.0	33.1	51.7
Western District*	1945	64.7		
Western District†	1947-48	45.7	29.8	52.1
Leitchville	1945	63.9		
Leitchville	1947-48	50.0		
King Island	1945	69.0		

* Allansford; † Allansford and Garvoc.

The results obtained in 1947-48 are generally comparable with those of Barnicoat (1947) obtained 12 years earlier in New Zealand, where similar dairying conditions exist. They also confirm those of Kunerth and Riddell (1938) who found very low vitamin A potency in fat produced by cattle on drought-stricken pasture. It is usual, of course, in northern countries to associate low vitamin A values with winter butterfat. Our results shown the vitamin A

potency in winter butterfat to be well up and approaching the maximum because of the availability of green, though slow growing, pastures after the autumn rains. Das Gupta (1937) has recorded a similar state of affairs in Bengal, though in this case the cattle are denied pasture in the summer because of wet season flooding.

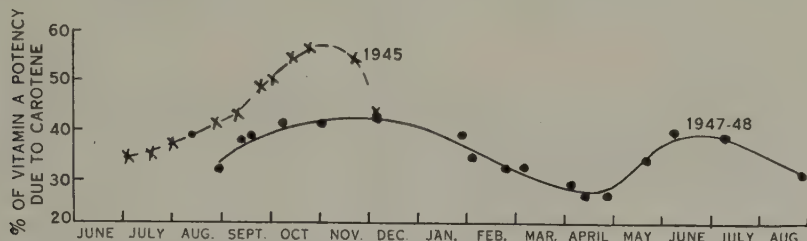


Fig. 6.—Seasonal fluctuations in the percentage of vitamin A potency due to carotene in Allansford butterfat.

It is noteworthy that the carotene figures are more constant than the vitamin A throughout the year. The percentage of total vitamin A potency due to the carotene has been graphed for Allansford (Fig. 6) and Drouin (Fig. 7). Two peaks are apparent; one in summer and one in winter. The former corresponds roughly with falling total vitamin A potency and the latter with rising total potency. The minima occur in late winter or early spring and in the autumn.

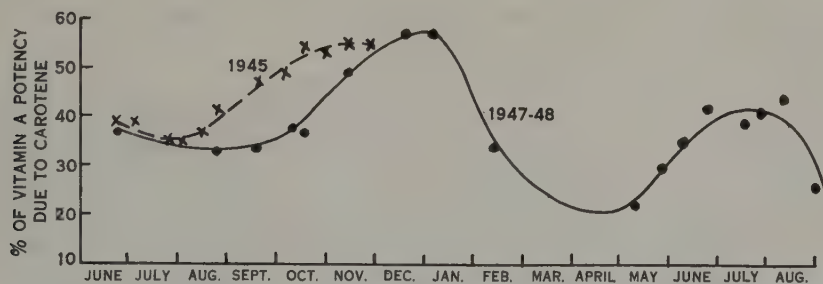


Fig. 7.—Seasonal fluctuations in the percentage of vitamin A potency due to carotene in Drouin butterfat.

The proportion of total potency due to carotene rises above 50 per cent., is usually 35 to 40 per cent., and sometimes below 35 per cent. This is a much higher proportion than is reported by Ronnenberg (1945) for Danish butterfat (about 20 per cent.), by Jenness and Palmer (1945) for Minnesota butter (11 to 15 per cent. in winter, 21 to 25 per cent. in summer), or by Parrish *et al.* (1946) in Kansas butter (14.9 per cent. in winter, 21.4 per cent. in summer). However, Barnicoat (1947) shows comparable figures for New Zealand butterfat; 37-42½ per cent. for Manawatu and 34 to 52 for Waikato. This phenomenon can probably be related to the carotene-rich diet of continuous pasture.

It will have been noticed that the 1945 values are much higher than those for 1947-48. These figures were supplied to Dr. F. W. Clements of the Institute of Anatomy, Canberra, in 1946, and formed the basis of the vitamin A figures in Australian butter and cheese published by Osmond (1946). A critical study of the methods and standards, etc. used in the two phases of the work failed to reveal any anomalies and an explanation of the phenomenon was sought in the climatic conditions obtaining in the two periods and in the statistics of milk production.

Table 2 records for Victoria the total dairy cattle for the year and the milk produced in the period June to December for the past four years as set down in the "Summary of the Dairy Industry in Australia," an annual publication of the Commonwealth Statistician.

TABLE 2
TOTAL DAIRY CATTLE AND MILK PRODUCED IN VICTORIA

Year	Total Dairy Cattle (thousands)	Milk Produced from June- December (millions of lb.)
1945	1364	2013
1946	1299	2777
1947	1411	2728
1948	1476	2758

In 1945 a slightly smaller number of cattle produced in the crucial months little more than two-thirds as much milk as in 1947 and 1948. The 1945 season followed a comparatively dry autumn.

Table 3 shows the percentage of the mean average rainfall received in each period in the three Victorian districts in the years under discussion and the pasture notes of the Commonwealth Meteorological Bureau, Melbourne, refer to unsatisfactory pastures in June 1945, "slow," "retarded," and "fair" pastures in all areas (including King Island) in July and August and good growth but accompanied by frosts in September in all three Victorian areas. This slow start would account for the lower milk production for the June-December period in 1945 compared with the same periods in 1947 and 1948 when rainfall was more nearly normal, and the pasture notes show the satisfactory development of pastures with no abnormalities. Actually milk production for the whole 1945-46 season was almost normal as the season "held on," the number of cows in milk on March 31, 1946 being abnormally high.

It is submitted that the higher vitamin A figure for the 1945 "flush" can be attributed to the lower volume of milk in that the cattle on the slow but green pastures ingested normal quantities of carotene which then appeared in a smaller volume of milk, leading to a high vitamin A potency in the fat actually produced. This is in fact a concentration effect.

It is well known that when the rest of Victoria is dry and parched in late summer the Snowy River flats at Orbest in the far east of the State retain an

abundance of green feed. On King Island, too, with a more even summer temperature and absence of drying north winds, the pastures usually last better. It seemed, therefore, that a comparison of vitamin A potency of February butterfat (corresponding with the minima in the curves in Figs. 1, 2, 3) from the different areas mentioned would reveal higher values for Orbost and, possibly, King Island. A detailed study was not possible, and isolated samples have been assayed.

TABLE 3

RAINFALL IN YEARS AND DISTRICTS STUDIED EXPRESSED AS PERCENTAGE OF AVERAGE MEAN RAINFALL FOR THE PERIODS SHOWN

	Jan.-Mar.	Apr.-July	Aug.-Dec.
1945			
Allansford	124	41	85
Drouin	80	78	88
Cohuna (Leitchville)	45	106	101
King Island	90	80	123
1947			
Allansford	145	101	134
Drouin	127	112	126
Cohuna (Leitchville)	138	112	167
King Island	148	160	138
1948			
Allansford	54	111	105
Drouin	52	124	101
Cohuna (Leitchville)	30	175	100
King Island	64	138	115

Table 4 compares the vitamin A potency of February butterfats from six dairy products factories in five areas. Leitchville, Drouin, Allansford, and Garvoc samples were from cheese manufactured during the first week in February; King Island and Orbost from butter manufactured in the third week.

These results point to the superiority of the Orbost butterfat over that from the other areas so far as vitamin A potency is concerned and strikingly confirm the influence of feed on this property. Although there is only one result from the Orbost district, it seems likely from what is known of seasonal variation and the influence of fresh pasture on vitamin A potency of butter that there is no serious seasonal fluctuation in this area. While so few results cannot be regarded as conclusive, they are in line with the recorded facts.

It is noteworthy that the values for Gippsland and Western District butterfats are higher than those obtained at the corresponding period in 1948; 37 as against 30 I.U./g. for Allansford and 40 as against 33-34 for Drouin. This is probably due to the abnormally low rainfall in the first quarter of 1948 (see Table 3) and further serves to underline what has already been noted — that the vitamin A potency of butterfats from the same area at the same period

each year may be subject to significant fluctuations. This must follow from the prosperity of the season, but may be a much greater fluctuation than hitherto imagined.

TABLE 4
VITAMIN A POTENCY OF LATE SUMMER BUTTERFAT, FEBRUARY 1949

Area and Factory	Vitamin A Potency, I.U./g.		Total
	Vitamin A	Carotene	
King I. (Loorana)	23.4	11.0	34.4
King I. (Loorana)	28.0	11.0	39.0
Murray Irrigation (Leitchville)	21.0	18.3	39.3
East Gippsland (Orbost)	34.3	19.8	54.1
West Gippsland (Drouin)	24.0	15.6	39.6
Western District (Allansford)	24.0	13.1	37.1
Western District (Garvoc)	22.0	12.5	34.5

In Table 5, average values for vitamin A in Victorian butter are compared with values recorded in the literature.

TABLE 5
COMPARISON OF VITAMIN A POTENCY OF BUTTER OF DIFFERENT COUNTRIES

Location	I.U./lb.	Authority
U.S.A.; Washington	8,700 to 25,900	Ashworth <i>et al.</i> 1945
U.S.A.; Kansas	11,050 to 17,700	Parrish <i>et al.</i> 1946
U.S.A.; Minnesota	9,000 to 17,000	Jenness and Palmer 1945
U.S.A.; Wisconsin	9,500 to 18,000	Berl and Peterson 1944
U.S.A.; Texas	17,000	Kemmerer and Fraps 1943
Denmark	20,700	Ronnenberg 1945
Denmark	7,000 to 17,100	Wilkinson 1939
Scotland	5,700 to 12,180	Wilkinson 1939
England	4,600 to 18,200	Morgan and Pritchard 1937
Norway	9,000 to 17,000*	Hvidsten 1943
New Zealand	13,200 to 18,600	Barnicoat 1947
Australia; Victoria 1945	Up to 29,000*	---
Australia; Victoria 1947-48	11,000 to 21,000*	---

* Calculated as 80 per cent. of the values obtained for dry butterfat.

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ELECTRON MICROSCOPIC STUDIES OF SPERMATOOZOA

II. THE MORPHOLOGY OF THE HUMAN SPERMATOZOON

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Summary

Suitable washing techniques, which do not appreciably affect sperm motility, and enzymic digestion, have made it possible to give a clear picture of the finer structures in the human spermatozoon, using the electron microscope.

The axial filament, which arises from the anterior distal centriole, consists of nine or possibly eleven fibrils, which have a maximum diameter of about 500 Å, and taper gradually towards the tip of the tail. The proximal regions of the fibrils, immediately adjacent to and within the anterior distal centriole, consist of a number of granules, about 600×400 Å, longitudinally aligned. These granules are presumably concerned in the protein synthesis which is responsible for the growth of the axial filament during spermiogenesis.

The axial filament is surrounded in the mid-piece of the sperm by a broad helically-wound cord or spireme, which terminates, after 12-15 turns, at the annular posterior distal centriole.

In the sperm tail, the axial filament is encased in a strong sheath, the major component of which is a closely-wound helical cord about 200 Å in diameter, ending abruptly about 7-10 μ from the distal extremity of the axial filament. In marked contrast to the behaviour of bull sperm, this naked tip of the axial filament does not readily fray into the component fibrils.

The tail contains two fibrils which appear to be less susceptible to pepsin than the remainder. This result seems to imply some functional differentiation, and the mechanism of sperm locomotion is discussed in the light of the structural detail revealed.

I. INTRODUCTION

In recent years a number of reports on the electron microscopy of spermatozoa have appeared in the literature. These include studies on bull and fowl sperm (Baylor, Nalbandov, and Clark 1943), fowl sperm (Grigg and Hodge 1949), bull sperm (Bretschneider and Van Iterson 1947) and *Arbacia* sperm (Harvey and Anderson 1943). Schmitt (1944) has discussed the ultra-structure of sperm tails in the light of the electron microscopical and optical evidence.

The human spermatozoon, however, has received little attention from electron microscopists, possibly because it does not lend itself so easily to direct methods of examination. Only two reports have appeared to date. Seymour and Benmosche (1941) described the general appearance of the human spermatozoon and noted that the mid-piece was apparently segmented. Reed

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and Reed (1948) examined spermatozoa from 38 young healthy subjects. They found that the galea capitis and brush-branching of the tail commonly observed in the bull sperm were characteristically absent in the human spermatozoon, and claimed that there was evidence for a spiral formation within the mid-piece. This resembles the finding of Bretschneider and Van Iterson (1947) that there is a helically-wound cord in the mid-piece of the bull sperm.

In the work reported here, the application of enzymic digestion techniques has enabled the finer structural details to be elucidated more fully than in the past. These methods have already been used to advantage in studies of the fowl spermatozoon (Grigg and Hodge 1949).

II. MATERIALS AND METHODS

The relatively high proportion of colloidal and other extraneous material present in fresh human semen rendered it unsuitable for the mounting of specimens for electron microscopic (E.M.) examination without further treatment. Dilution with distilled water and washing by centrifugation was a useful technique in separating the spermatozoa from the colloidal material also present in the semen, but, in view of the drastic effects of distilled water on fowl spermatozoa (Grigg and Hodge 1949), there is necessity for caution in the interpretation of the results obtained by this method. The work on fowl spermatozoa has made it clear that the safest preparative procedures for spermatozoa are, in general, those in which the motility is not impaired by the treatment. The motility is thus a convenient index of the structural integrity of the spermatozoa being treated. Suitably clean suspensions of human spermatozoa were easily obtained by the procedure outlined below, and one could be certain that the spermatozoa were free from any distortion or structural alteration, since the motility was not appreciably impaired at any stage of the washing procedure.

Samples of fresh semen were diluted with 15-20 volumes of Tyrode solution and agitated to ensure homogeneous dispersion. There was no apparent decrease in motility arising from the dilution, a result which is in marked contrast to the rapid cessation of all movement which occurs if the semen is diluted with distilled water. Thermal shock effects were guarded against in all manipulations by the avoidance of sudden temperature changes. The spermatozoa were spun down in an angle centrifuge at 1000-2000 r.p.m. and resuspended in fresh Tyrode solution after discarding the supernatant liquid. The centrifugation and resuspension was repeated three or four times in order to free the sperm from as much contaminating material as possible, the motility being checked after each operational sequence. After five centrifugations, the majority of the spermatozoa were still highly active, and any loss in motility could be attributed to the mechanical effect of centrifugation. The adequacy

of the procedure is indicated by the observation that many of the spermatozoa in a thrice-washed suspension were still active after 48 hours storage at room temperature.

The spermatozoa could now be mounted by allowing a drop of the suspension to dry on a conventional specimen screen covered by a collodion film. However, this procedure resulted in the formation of numerous salt crystals, so that there was some danger of distortion. There was also the possibility of structural changes due to the increasing hypertonicity during evaporation of the drop to dryness. Although salt crystals were easily removable by washing the screen in distilled water, this involved the possibility of drastic alterations as a result of exposing the spermatozoa to a hypotonic medium. These dangers were effectively overcome by adding a small volume of 10 per cent. formalin to the final washed suspension to bring the concentration of formalin to about 0.5 per cent. All movement ceased immediately and suspensions were then left overnight to ensure complete fixation, spun down and resuspended in distilled water. One or two washings sufficed to obtain a salt-free suspension from which specimens for examination in the electron microscope were easily prepared. Formalin-fixed spermatozoa were free from the disruptive effects of distilled water. The above technique of washing in suitable isotonic media, adding formalin, and finally washing in distilled water seems to be generally applicable to biological materials which it is desired to obtain free from the structural alterations resulting from hypo- or hypertonicity of the suspending medium.

Tryptic* and peptic digestion was carried out on the final washed suspensions in distilled water. Several washings were necessary to remove all traces of free formalin. The spermatozoa were spun down, resuspended in a suitable buffer solution (pH 3 for pepsin, pH 8 for trypsin), and a few crystals of the enzyme added. With pepsin, 30 minutes at 37°C. was sufficient to break down most of the spermatozoa to a state suitable for examination. For trypsin much longer periods were necessary (several days). Enzymic action was stopped after the required incubation period by the addition of formalin, and the material either mounted immediately or after further washing with distilled water.

The well-known technique of staining specimens with phosphotungstic or phosphomolybdic acid was often employed as a means of increasing image contrast, as was the shadow-casting technique of Williams and Wyckoff (1946). Platinum was used exclusively as the shadowing metal in preference to gold, which tends to migrate and coagulate under the influence of a high-intensity electron beam. In all shadow-cast specimens, the shadow-casting was carried out so that the ratio of shadow length to object height was about 4:1.

The electron microscope used in the present investigation was an R.C.A. Type EMU. The instrument was fitted with an objective aperture and was calibrated for magnification by the method of Farrant and Hodge (1948).

* The enzymes used in the investigation were "Difco" pepsin and "B.D.H." trypsin.

III. RESULTS

(a) General Appearance

Plate 1, Figures 1 and 2, illustrates the outline of spermatozoa after the Tyrode-formalin treatment described above. The sperm head varies both in size and shape, but is usually ovoid in outline and $3\text{--}4\mu$ long. Although some of this variation in appearance is due to individual differences, much of it is the result of the manner in which the sperm lie on the supporting film. That many of the sperm heads do not lie flat on the film but project upwards at various angles has been demonstrated by shadow-casting. The sperm head is attached to the mid-piece and tail by a short region ($<1\mu$) which may be termed the neck. This region corresponds to the articular strands described by Bretschneider and Van Iterson (1947) in bull sperm. The neck is immediately followed by the mid-piece, which is $0.6\text{--}0.7\mu$ wide and $5\text{--}6\mu$ long, and often shows internal structure, which will be considered in detail later. The mid-piece is followed by the tail proper, which is about 0.5μ wide at the tail-mid-piece junction and tapers gradually until a point about $7\text{--}10\mu$ from the extreme end is reached. At this point the width decreases abruptly and remains fairly constant up to the tip (Plate 1, Figs. 2 and 4; Plate 2, Fig. 6). Plate 2, Figure 6, shows that this break in outline corresponds with the termination of the helically-wound cord of the tail sheath. The naked tip of the tail ($7\text{--}10\mu$ in length) is usually intact and shows no sign of fraying. This is in agreement with the observation of Reed and Reed (1948) that there is no brush branching of the tail in this region. Occasionally, however, fraying does occur as a result of "osmotic shock" (Plate 2, Fig. 5).

None of the sperm examined showed the presence of the galea capitis, which is normally present in bull sperm, nor was there any sign of the crater-like notch on the vertex of the head, which Seymour and Benmosche (1941) suggested was possibly some form of suction apparatus to facilitate penetration of the ovum.

The overall lengths of the spermatozoa were between 50 and 65μ . Here again, as for the sperm head, there was a considerable range of individual variation. A number of sperm had cytoplasmic beads attached at various points, but most frequently on the mid-piece close to the head. These beads varied in size up to something approaching the dimensions of the sperm head. Their presence did not seem to affect the motility of the spermatozoa as seen in hanging-drop preparations. Although a few spermatozoa were observed with small heads and other possibly abnormal features, the description of such abnormalities is beyond the scope of the present paper, which is intended only as a basis of reference for future work on the development and pathology of spermatozoa. It is believed that the features described here are those of normal spermatozoa.

(b) The Sperm Head

The profile of the sperm head is shown in Plate 1, Figure 3, which was taken when the supporting film ruptured and curled over. The thickest part of the head is about 1μ . It will be seen that the anterior region of the head is thin, and tapers towards the anterior extremity. The thinness of the head here explains why there is often a region which is partially transparent to 50 kilovolt electrons. The high density of the material in the head is consistent with the belief that it consists mainly of nuclear material. It seems reasonable to suppose that Plate 1, Figures 1 and 2, presents fairly good representations of the appearance of the spermatozoa in their natural state, provided that some allowance is made for the moderate degree of flattening arising from the dehydration necessarily imposed by electron microscopic examination. Dawson and MacFarlane (1948) and Farrant and O'Connor (1949) have discussed the dimensional changes resulting from drying, under various conditions, of the larger animal viruses. Their results appear to indicate that fixation, in general, has a beneficial effect in minimizing the partial collapse which occurs on dehydration of biological material.

Pepsin appears to have little effect on the material of the sperm head (Plate 2, Fig. 7; Plate 5, Fig. 14). The heads are swollen by trypsin and often show transparent areas. Occasionally they burst and the material within is extruded, but it has not yet been possible to demonstrate the chromatin fibrils which are clearly visible in fowl sperm heads after similar treatment (Grigg and Hodge 1949). Swelling of the head, similar to that observed in trypsin-treated samples, was also observed when spermatozoa, after repeated washing in Tyrode solution, were spun down and resuspended in distilled water.

(c) Mid-Piece and Tail

These two structures seem to be differentiated regions of a more general structure which might be termed the locomotor unit. The justification for considering them together lies in the observation that the axial filament runs continuously throughout both structures from the rear of the head to the tip of the tail, an organization which implies a physiological as well as a structural continuity.

Plate 2, Figure 7; Plate 3, Figure 8; Plate 4; Plate 5; Plate 6; and Plate 7 illustrate the internal structure revealed by treatment with pepsin at pH 3. The axial filament evidently consists of a number of fine fibrils (Plate 4; Plate 5; and Plate 6, Fig. 15) and extends continuously throughout the mid-piece and tail. In the mid-piece region, the axial filament appears to consist of nine fibrils (Plate 5, Fig. 13; Plate 6, Fig. 15), none of which are differentiated in appearance or resistance to peptic digestion from the remainder. The interpretation of the results for the tail region is complicated by the occurrence of two dense fibrils which appear to be intimately associated with the helical cord of the tail sheath (Plate 4, Fig. 12; Plate 7, Fig. 18). Unfortunately it has not been

possible to determine whether these two fibrils are additional to the nine already demonstrated, or whether they indicate some differentiation in the tail region of two of the nine fibrils; that is, whether the tail contains a total of eleven or nine fibrils respectively. It is of interest to note in this connection that Grigg and Hodge (1949) have demonstrated a total of eleven fibrils in the fowl sperm tail in which the membranous tail sheath is easily disrupted by distilled water. Two of these fibrils are clearly differentiated from the remaining nine on the basis of their relative susceptibility to distilled water and peptic digestion. In the human spermatozoon the presence of a helical cord in the tail sheath prevents fraying of the tail in distilled water, but it seems likely that there is a similar differentiation among the tail fibrils.

The axial filament is surrounded in the mid-piece by a helical structure (Plate 2, Fig. 7; Plate 3, Figs. 8 and 9), which has often been referred to in the literature as the spireme, and is believed to be derived from the mitochondria which migrate to this region during spermiogenesis. Plate 4, Figures 11 and 12, shows the mid-piece and tail in spermatozoa which have suffered extensive peptic digestion. The latter Figure demonstrates clearly that the spireme breaks down into a number of elongated bodies, which in the intact spermatozoon are presumably joined end to end to form a continuous helical cord. It is of interest to note that in the fowl spermatozoon the mitochondrial granules are apparently arranged to give a true segmentation, and there is no helical winding in the mid-piece or tail (Grigg and Hodge 1949). Plate 3, Figure 10, illustrates the helical arrangement in the mid-piece of the bull sperm. The three fine filaments, which stain densely with phosphomolybdic acid, appear to be components of the broad helical cord described by Bretschneider and Van Iterson (1947). Similar fine filaments have been observed in the mid-piece of the human spermatozoon, particularly on the original plates, but were not sufficiently clear for reproduction in this paper. Since helical structures have also been observed in the mid-piece of ram and marsupial sperm (Hodge, unpublished data), the evidence available at present seems to indicate that helical structures are characteristically present in the mid-piece of mammalian spermatozoa.

The fibrils of the axial filament are surrounded in the anterior portion of the tail proper by a strong sheath, the major component of which is a helically-wound cord about 200 Å in diameter. This helical winding may be seen in untreated sperm (Plate 1 and Plate 2, Figs. 5 and 6), but is most clearly visible in preparations subjected to peptic digestion (Plate 6, Fig. 16; Plate 7, Fig. 17). It commences at the tail-mid-piece junction (Plate 2, Fig. 7) and terminates at a point 7-10 μ from the extreme tip of the tail (Plate 2, Fig. 6). This helical cord has also been demonstrated in bull sperm (Bretschneider and Van Iterson 1947) and in marsupial sperm (Hodge, unpublished data). It is not present in avian sperm (Grigg and Hodge 1949) or in *Arbacia* sperm (Harvey and Anderson 1943). It seems reasonable, therefore, to suppose tentatively that it is a characteristic feature of mammalian sperm, although more extensive

investigation will be necessary before this view can be definitely accepted. The presence of the helical cord affords an explanation of the failure of mammalian sperm tails to fray into fibrils when placed in distilled water. This phenomenon occurs easily with fowl and squid sperm, which have only a membranous tail sheath, easily ruptured by "osmotic shock."

The failure of the naked tip of the human sperm tail to fray as does the tip in bull sperm seems capable of two explanations. The axial filament itself may be surrounded by a relatively tough membrane or the fibrils may be effectively cemented together by an inter-fibrillar matrix material. Such a matrix material has been demonstrated in the fowl sperm, but evidently fails in some degree to prevent separation of the fibrils, since the tails fray easily in distilled water.

There is little to suggest why the helical winding should end abruptly, leaving about 8-10 μ of naked axial filament. It seems possible that there is here a functional analogy with such locomotor organs as the fish tail, where as is well known, the "flexibility" increases steadily as the extremity of the tail is approached. A consideration of the hydrodynamic principles involved shows that such a variation in "flexibility" is essential if the propulsive organ is to work at maximum efficiency. This variation is apparently partially achieved in the sperm by the tapering of the tail from the tail-mid-piece junction. That the propulsive movements of the sperm tail are essentially similar to those of the fish tail is easily seen in hanging-drop preparations in which the sperm have lost most of their initial vigorous activity.

The helical cord itself shows signs of a characteristic fine structure (Plate 7, Fig. 17), which has however been obscured to some extent by the drastic procedures required to free the helical cord from the axial filament. The functional nature of this cord is obscure. That it is not essential for locomotion is indicated by its absence in many other types of sperm. It would seem to be primarily a structural feature, but may possibly contribute in some degree towards efficiency of propulsion.

The fibrils of the axial filament arise from a composite body (Plate 5, Fig. 13, Plate 6, Fig. 15) which will be termed the anterior distal centriole in accordance with the nomenclature adopted for the fowl spermatozoon (Grigg and Hodge 1949). The anterior distal centriole is located at the rear extremity of the sperm head, and according to classical observations on spermiogenesis is immediately adjacent to the proximal centriole in the mature sperm. The axial filament is believed to develop from the anterior distal centriole during spermiogenesis. None of the electron microscope evidence is at variance with this belief, but a systematic study of spermiogenesis with this instrument would be necessary to confirm it.

The fibrils of the axial filament are about 500 Å wide in the vicinity of the anterior distal centriole and taper gradually to a relatively uniform diameter in the tail proper. The anterior extremity of each fibril consists of several longitudinally aligned granules about 600×400 Å (Plate 7, Fig. 18). These

small granules are possibly concerned in the production of the fibrils during spermiogenesis. The anterior distal centriole seems to consist almost exclusively of them.

The tail fibrils sometimes exhibit what appears to be a periodic cross-striation, the axial period being about 100 Å. This structure appears to be similar to that observed by Hall, Jakus, and Schmitt (1945) in smooth molluscan muscle, but peptic digestion obscured the structure to such a degree that reproduction of the micrographs was impossible. However, a structure such as this is not unlikely, since all evidence to date suggests that these fibrils must be contractile elements responsible for sperm locomotion. The thinness of the fibrils in the region beyond the mid-piece in pepsin-treated sperm (Plate 4, Fig. 11) suggests that they are composite structures, consisting of an outer easily digested layer surrounding an inner more resistant core, and moreover, that there is some differentiation between the part of each fibril contained within the mid-piece and that part within the tail itself, at least with respect to their resistance to enzymic attack. This may well be a reflection of some functional difference. A similar diminution in diameter of the tail fibrils has also been observed in fowl sperm after enzymic digestion (Grigg and Hodge 1949).

The possible roles of the two well-defined fibrils seen in Plate 4, Figure 12, and Plate 7, Figure 18, are of considerable interest. These fibrils appear to be relatively insusceptible to the action of pepsin as compared with the other fibrils in the tail, a phenomenon which suggests that there may also be a functional differentiation between the two types of fibrils. Any theory regarding their function must of necessity at this stage be purely speculative, but it seems worthwhile to point out that if the fibrils of the axial filament are contractile elements responsible for sperm locomotion, then the characteristically "sinusoidal" flexure of the sperm tail during motion must be induced by localized contraction of small lengths of the fibrils in a regular time sequence pattern. The "sinusoidal" configuration of the tail cannot be explained by a sequence of contractions in which the whole length of each fibril is simultaneously involved, and it becomes apparent that only a fraction of any given fibril can be contracted at any given instant. If this is so, a "sinusoidal" flexure can only be achieved in two ways:

- (a) The contraction of a fibril may pass along its length in the form of a wave of contraction. If then waves of contraction are initiated in the various fibrils in some definite order and with a time-lag between the initiation of each pulse, a "sinusoidal" flexure could be obtained.
- (b) If the two differentiated fibrils (Plate 4, Fig. 12; Plate 7, Fig. 18) are regarded as "neural" fibrils, capable of transmitting impulses, then the localized contraction of the contractile fibrils may occur only in those regions in intimate contact with the "neural" fibrils. Thus if the "neural" fibrils cross over one another several times in the length of the tail and alternately transmit impulses in a regular time sequence, the tail must assume a "sinusoidal" configuration at any given instant.

Although the two differentiated fibrils were often observed to cross over one another, there was no apparent regularity and the observation may well have been fortuitous. The observation that the tail fibrils appear to be composite structures, consisting of a relatively resistant core and a less resistant sheath (see Plate 4, Fig. 11 and Plate 7, Fig. 18) seems to favour the notion of waves of contraction. Indeed this is the simplest possible explanation of the functioning of the axial filament, but leaves unexplained the structural complexity of the tail as a whole.

IV. ACKNOWLEDGMENT

The work described in this paper was carried out as part of the research programme of the Division of Industrial Chemistry, C.S.I.R.O.

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APPENDIX I

LIST OF PREPARATIVE TREATMENTS

(a) Fresh semen diluted with 15-20 volumes of Tyrode solution, centrifuged, and resuspended 3 X in Tyrode solution, formalin added, stored 24 hours at room temperature, centrifuged and resuspended 2 X in distilled water.

(b) Fresh semen diluted with 15-20 volumes of Tyrode solution, centrifuged, and resuspended 2 X in isotonic saline and 2 X in distilled water to produce "osmotic shock."

(c) Initial treatment as in (a), then acidified to pH 3 with HCl, pepsin added, incubated at 37°C., or 20°C. for 10, 20, or 30 minutes. (See captions to electron micrographs for time and temperature of incubation). Reaction was usually stopped with formalin and the samples centrifuged and resuspended 2 X in distilled water before mounting.

(d) Initial treatment as in (a), then resuspended in McIlvaine buffer at pH 8, trypsin added, incubated at 37°C. for 67 hours, reaction stopped with formalin, centrifuged and resuspended 2 X in distilled water before mounting.

EXPLANATION OF PLATES 1-7

Abbreviations used in electron micrographs.—h., sperm head; m., mid-piece; t., sperm tail; n., neck of sperm; a.f., axial filament; sp., spireme; a.d.c., anterior distal centriole; p.d.c. posterior distal centriole; t.s., tail sheath; t.h., termination of helical component of tail sheath; f., fibrils of axial filament; r.f., resistant fibrils; g., granules in fibrils of axial filament near anterior distal centriole.

PLATE 1

Fig. 1.—Human sperm treated as in (a) (see Appendix I for details of preparative treatments), shadowed with platinum, showing general appearance of head, mid-piece, and tail.

Fig. 2.—Complete human spermatozoon, treatment (a), shadowed with platinum.

Fig. 3.—Human sperm head, treatment (b), unstained, showing profile as seen after rupture and curling of the supporting film.

Fig. 4.—Human sperm, treatment (a), shadowed with platinum, showing typical appearance of the intact tail.

PLATE 2

Fig. 5.—Human sperm, treatment (b), unstained, showing tail and portion of mid-piece. Note fraying of the axial filament as a result of "osmotic shock."

Fig. 6.—Human sperm, treatment (a), stained with phosphomolybdic acid, illustrating the helical component of the tail sheath and its abrupt termination, leaving a length of the axial filament exposed.

Fig. 7.—Human sperm, treatment (c), incubated with pepsin for 20 minutes at 20°C., stained with phosphomolybdic acid, showing the broad helical structure within the mid-piece and the fine helical component of the tail sheath.

PLATE 3

Fig. 8.—Human sperm, treatment (c), incubated with pepsin for 30 minutes at 37°C., shadowed with platinum. Note helical structure in mid-piece and tail.

Fig. 9.—Human sperm, treatment (d) with trypsin, stained with phosphomolybdic acid. Note the fibrils of the axial filament running through the mid-piece and tail, and the helical structure in the mid-piece.

Fig. 10.—Bull sperm transported in yolk-citrate medium, centrifuged, and resuspended 6 X in Tyrode solution, formalin added, stored 24 hours at room temperature, centrifuged, and resuspended 2 X in distilled water, stained with phosphomolybdic acid, showing portion of mid-piece. Note the three densely-staining fibrils arranged in helical fashion around the fibrils of the axial filament.

PLATE 4

Fig. 11.—Human sperm, treatment (c), incubated with pepsin for 30 minutes at 37°C., stained with phosphomolybdic acid, illustrating the relative resistance of those portions of the fibrils contained within the mid-piece. Note also the granular appearance (g.) of the fibrils near the anterior distal centriole.

Fig. 12.—Human sperm, treatment (c), incubated with pepsin for 30 minutes at 37°C., stained with phosphomolybdic acid, showing disorganization of the helical structure in the mid-piece into a number of elongated granules and the two resistant fibrils in the tail. Note also the remains of the helical winding of the tail sheath.

PLATE 5

Fig. 13.—Human sperm, treatment (c), incubated with pepsin for 30 minutes at 37°C., shadowed with platinum, showing anterior distal centriole and fibrils of the axial filament.

Fig. 14.—Human sperm, treatment (c), incubated with pepsin for 30 minutes at 37°C., stained with phosphomolybdic acid. Note fibrils arising from the anterior distal centriole. Mid-piece has been entirely removed except for the axial filament.

PLATE 6

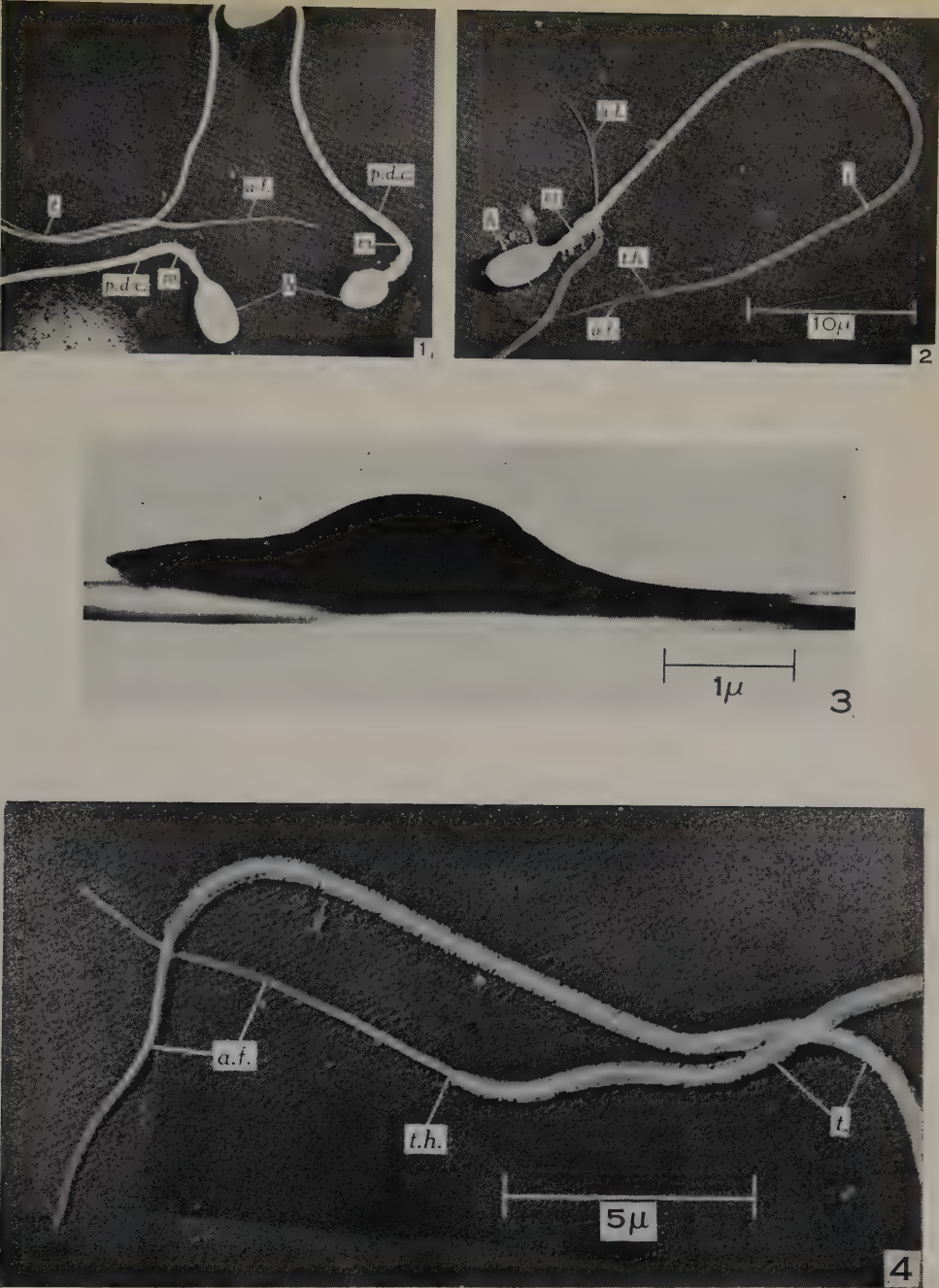
Fig. 15.—Human sperm, treatment (c), incubated with pepsin for 30 minutes at 37°C., shadowed with platinum, showing nine fibrils of the axial filament arising from the anterior distal centriole and passing into the tail sheath.

Fig. 16.—Human sperm, treatment (c), incubated with pepsin for 10 minutes at 20°C., stained with phosphomolybdic acid, showing in more detail the helical component of the tail sheath.

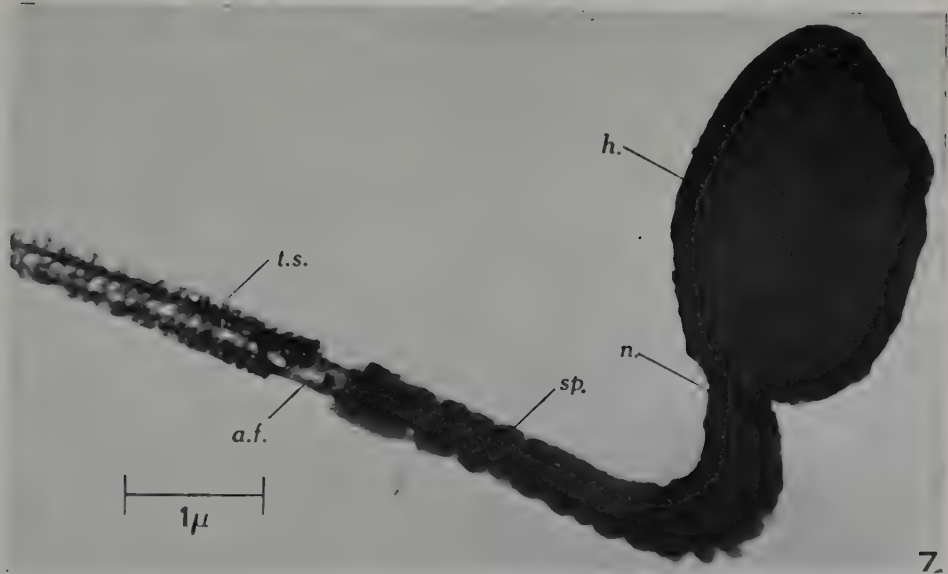
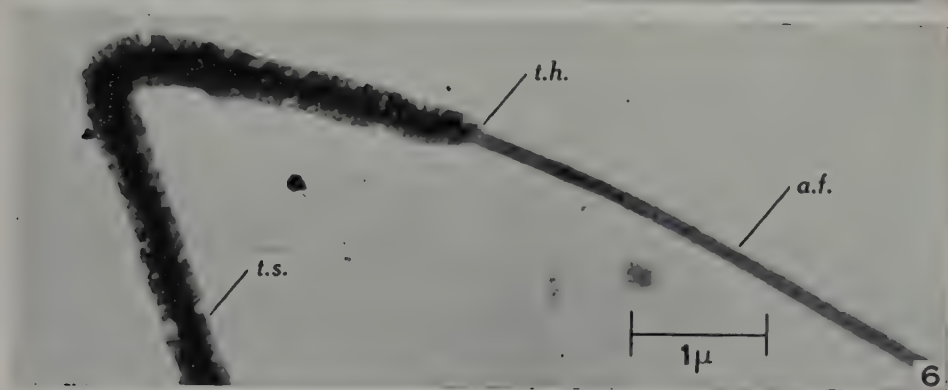
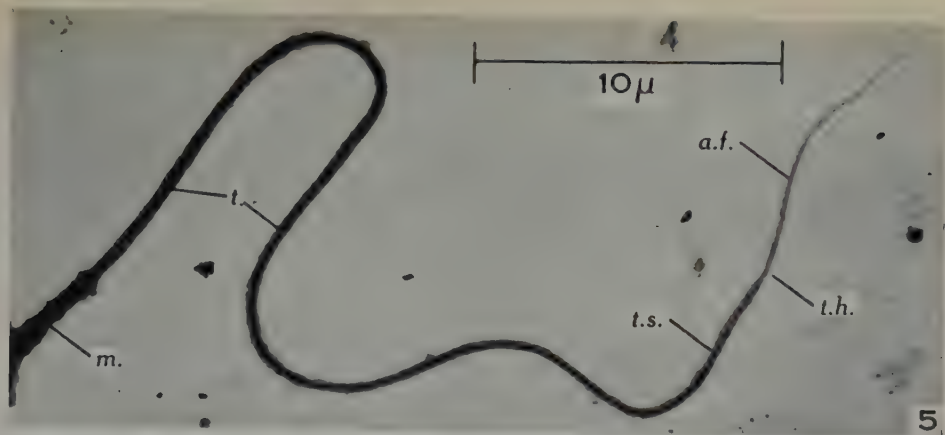
PLATE 7

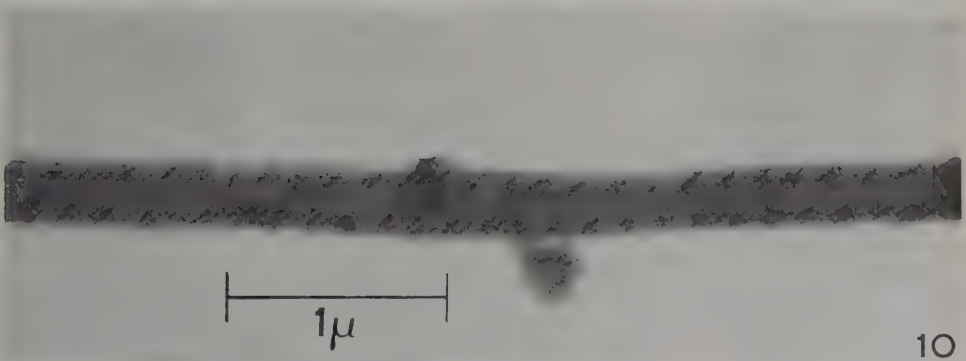
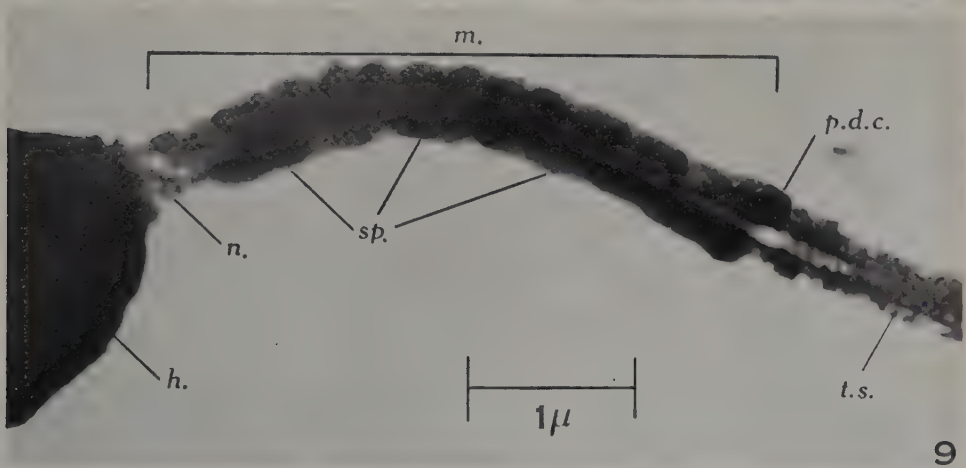
Fig. 17.—Human sperm, treatment as in Plate 6, Figure 16, stained with phosphomolybdic acid, small portion of the helically-wound component of the tail sheath. Note the fine structure of the helical cord.

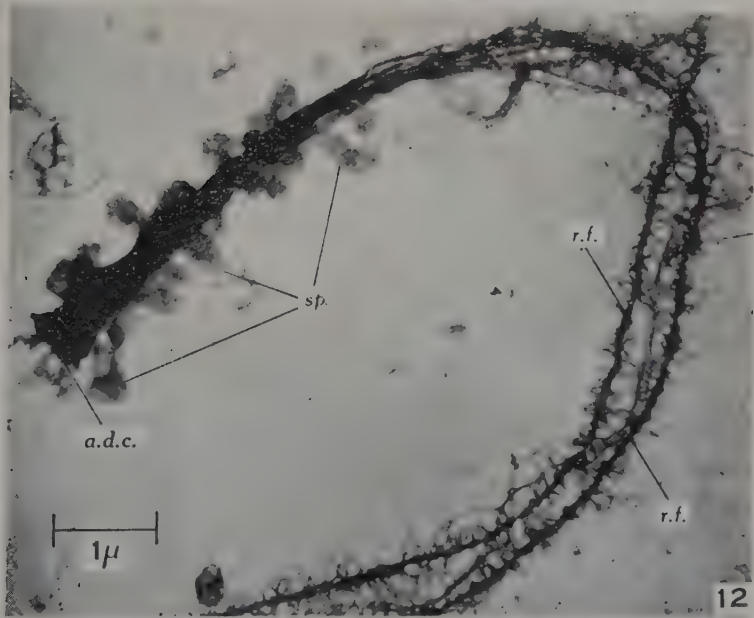
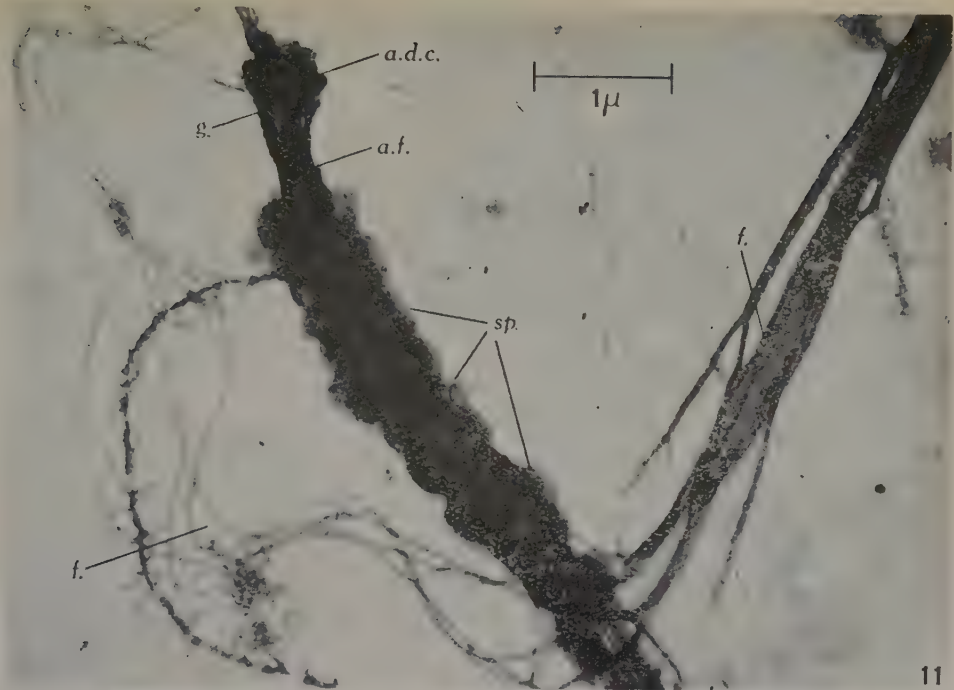
Fig. 18.—Human sperm, treatment (c), incubated with pepsin for 30 minutes at 37°C., stained with phosphomolybdic acid, showing the fibrils of the axial filament, granules (g.) adjacent to the anterior distal centriole, and the two resistant fibrils (r.f.).



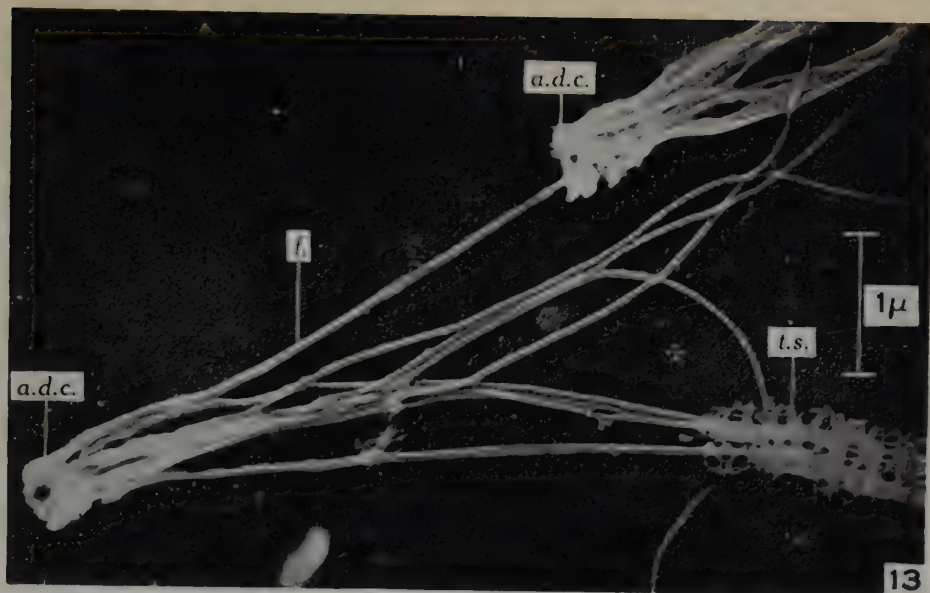
HODGE.—ELECTRON MICROSCOPIC STUDIES OF SPERMATOOZA

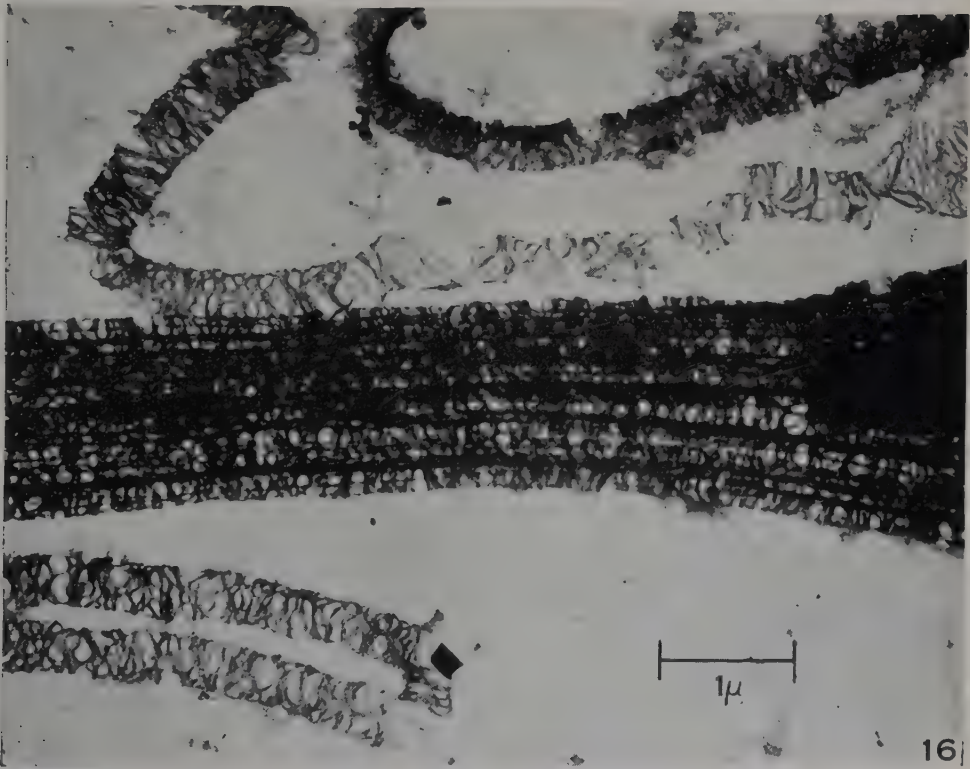
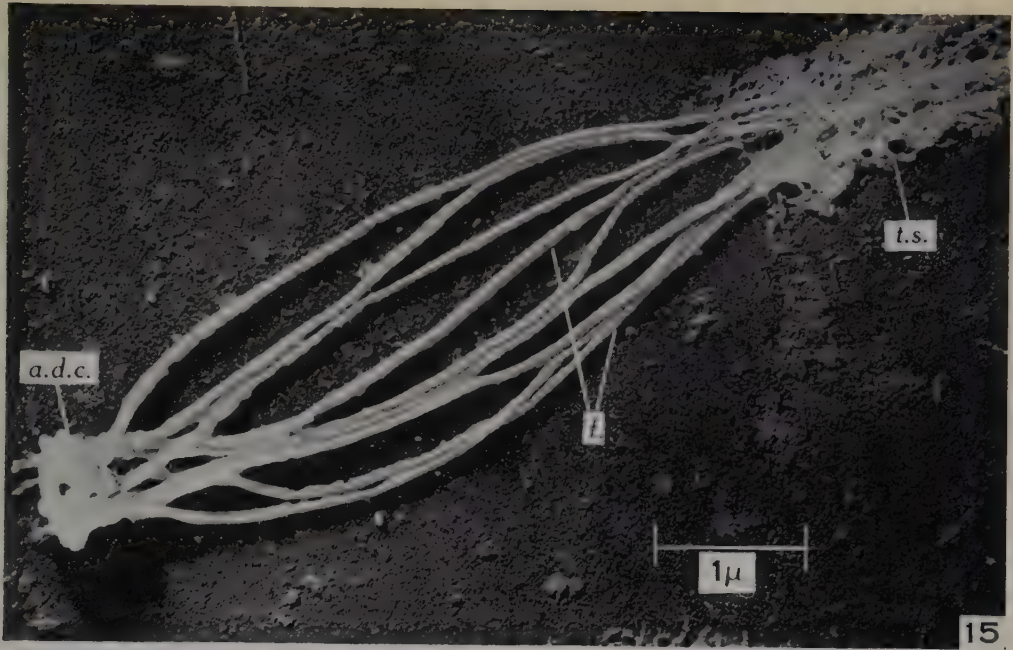


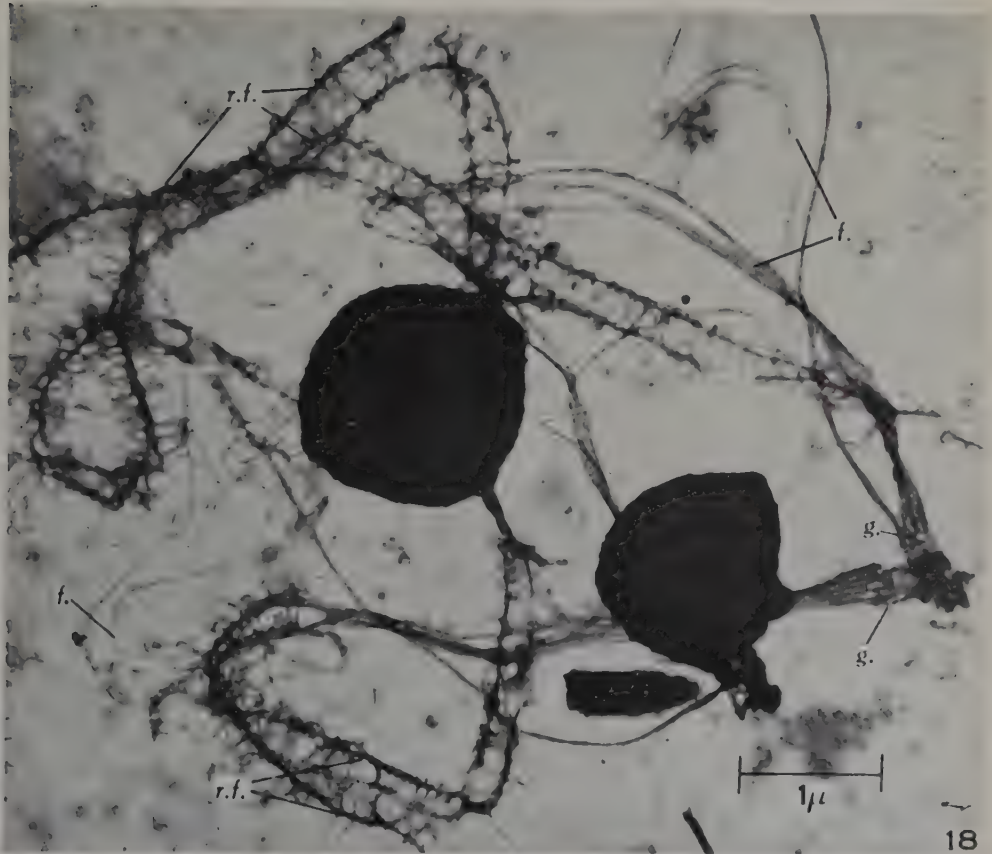
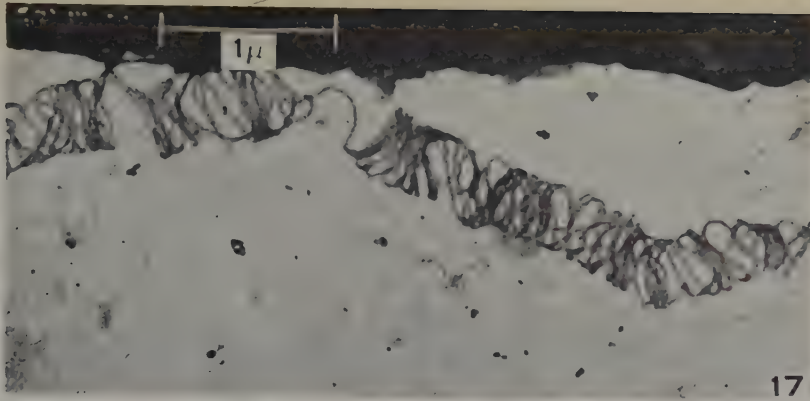




HODGE.—ELECTRON MICROSCOPIC STUDIES OF SPERMATOOA







HODGE.—ELECTRON MICROSCOPIC STUDIES OF SPERMATOOA

ON THE VEGETATIVE FORMS AND LIFE HISTORY OF *CHLOROMYXUM THYRSITES* GILCHRIST AND ITS DOUBTFUL SYSTEMATIC POSITION

By A. G. WILLIS*

(Plates 1-3)

[Manuscript received July 14, 1949]

Summary

The association between a myxosporidian parasite, *Chloromyxum thyrsites*, and "milky" specimens of the gempylid fish *Thyrsites atun* has been previously described by Johnson and Cleland (1910) and Gilchrist (1924). This association has been again confirmed in the present work with the addition of observations, from post-mortem studies, which show that there is a close correlation between the presence of aggregates of the parasite and the progressive liquefaction of the somatic muscular tissues (the condition of "miliness" which may affect from 5-7 per cent. of the commercial catches of *Thyrsites atun*).

The suggestion made by Gilchrist (l.c.) that the trophozoite of *C. thyrsites* is a uninucleate organism, in contrast to the multinucleate plasmodium found in all other species of the genus, has been confirmed in the present work by the discovery of the stages in sporulation intermediate between the trophozoite and the mature free spore. It has been found that the trophozoite is an irregularly shaped organism, with a single vesicular nucleus, which appears to reproduce for a variable period before the onset of sporulation. The sporulating trophozoite retains a single vegetative nucleus and may become mono-, di-, or polysporous. The spores are formed within sporogenous vacuoles which arise in the older trophozoites before sporulation. Each spore appears to develop directly from a binucleate sporoblast without the formation of a pansporoblast. The sporoblast passes successively through a syncytial stage with ten pycnotic nuclei and a cellular stage with ten vesicular nuclei. In the final transformation of the sporoblast into the mature spore, four nuclei, which appear to be closely associated with the formation of the spore membrane, degenerate and disappear.

Previous work on the structure of the spore has been largely confirmed, with the addition of some new observations on the structure of the spore membrane in media of low refractive index, and on the arrangement of the persistent nuclei of the capsulogenous cells.

Studies on the distribution of the parasite within its host have shown that it occurs as aggregated masses of either trophozoites, sporulating trophozoites, or spores. These masses lie within the somatic muscle fibres and not around them as described by Gilchrist (l.c.); each is composed of a variable number of organisms, the majority of which develop synchronously through the successive phases of the life history.

It is pointed out that the position of *C. thyrsites* in its present genus is anomalous in view of the absence of a plasmodial phase, and of a pansporo-

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blast, from its development. It is suggested, however, that reconsideration of the systematic position should be delayed until there is more information about the nature of the trophozoite and sporulation in forms like *C. histolyticum* and *C. quadratum* both of which resemble *C. thyrsites* in the possession of a quadrangular spore and the habit of muscular parasitism.

I. INTRODUCTION

The gempylid fish, *Thyrsites atun*, known popularly in Australia as the barracouta, occurs abundantly off the southern coasts of Australia, where it is of some commercial importance. This fish appears to be co-specific with the South African snoek. Commercial catches of both the Australian and South African forms have long been known to contain variable numbers of peculiarly diseased specimens, in which, from a few to twenty-four hours after capture, the flesh softens and finally disintegrates into a thick, viscous mass. Such fish are known as "milky barracouta" or "pap snoek" and they may constitute 5 per cent. of the catch in South Africa (Gilchrist 1924), or from 5-7 per cent. in Australia (Blackburn, unpublished data).

Until the work of Gilchrist (l.c.) the South African fishermen believed that "miliness" was the consequence of a bruising of the flesh due to inexperienced handling. Some years previously, however, Johnston and Cleland (1910) had demonstrated the presence of large numbers of myxosporidian spores in the flesh of the Australian "milky barracouta" and thus established the possibility that the degeneration was the result of a protozoan infection of the musculature. The structure of the spores led Johnston and Cleland to assume that they were produced by a species of the myxosporidian genus *Chloromyxum*. Gilchrist found spores of a similar type in "milky" specimens of the snoek and established a new species, *Chloromyxum thyrsites*, to include the spore forms observed by Johnston and Cleland and himself.

Johnston and Cleland did not observe the vegetative forms of *C. thyrsites*, and although Gilchrist made some interesting observations he did not demonstrate conclusively that the chains of rectangular, uninucleate bodies, which he assumed to be the vegetative organisms, had any genetic connection with the spores. Owing to the deficiencies in our knowledge of the vegetative life of the parasite, and of its life history, it has been impossible up to now to determine the exact relation which it bears to the muscular degeneration observed in "milky" fish, and to orientate methods of field research.

II. MATERIALS AND METHODS

The fact that our previous knowledge of *C. thyrsites* has been largely restricted to the spore is the result of certain difficulties in the way of obtaining properly fixed material from freshly caught, infected fish. The host, *Thyrsites atun*, is an oceanic fish which is normally caught by commercial fishermen only. Since these have no reliable method for discriminating between infected and

non-infected fish at the moment of capture, the only specimens to reach the hands of investigators on land have been disintegrated, "milky" fish, showing advanced sporulation of the parasites.

At the outset of the present work, the author was enabled to spend a week (February 15 to 22, 1949) on the Federal Research Vessel "Liawenee" for the purpose of barracouta fishing in Port Phillip Bay and Bass Strait. From the catches made in the vicinity of Barwon Heads, thirty freshly caught fish were selected at random. Slices, about 5 mm. thick, were excised from the somatic musculature of each selected fish and fixed in one of the following mixtures: aqueous Bouin, Carnoy, mercuric chloride-acetic acid (95 parts saturated aqueous solution of the former to 5 parts acetic acid). In addition, samples of the wall and contents of the alimentary canal were obtained from each fish and fixed in 5 per cent. formaldehyde-saline. After an interval of 5-6 hours each fish was sampled again, in exactly the same manner. The remains were then left overnight in order to determine any potentially "milky" fish. Two fish from among the thirty sampled turned "milky" overnight. From these were obtained fixed smears and casts made by squirting the decomposing flesh from a pipette into fixative.

The samples collected in the field were later embedded in wax and sectioned at various thicknesses. Thick sections ($18-25\mu$) proved to be of great value in the study of the trophozoite and spore, for they made it possible to examine these structures as a whole and in their true relationships to the tissues of the host.

Sections and smears were stained in Heidenhain's iron-alum haematoxylin, Ehrlich's haematoxylin and eosin, and Mann's methyl blue-eosin. Spores at various stages of maturity were also stained with a Lugol solution of iodine.

The drawings were made with an Abbé camera lucida in conjunction with a Leitz oil-immersion objective (ap. 1.30) and oculars 10 and 18.

The interrelation of the various stages described in the present paper has been demonstrated by the presence of the highly characteristic spore (with the spore aggregate and the sporulating trophozoites), and by the presence of some of the preliminary nuclear phenomena of sporulation (with the phases described as younger and older trophozoites). These criteria, applied to the materials available for the present work, lead to the conclusion that *C. thyrsites* was the only protozoan species present in the somatic musculature of the "milky" specimens; there was no trace of any metazoan parasite.

III. VEGETATIVE FORMS

According to Gilchrist (1924), *C. thyrsites* attacks the muscle fibres from the outside, and in one instance only was it found penetrating the muscle fibre (p. 271). Elsewhere (p. 269) he states that the trophozoites occur "... usually in long chains between the muscle fibre and the sarcolemma." Each chain is described as consisting of from ten to fifty uninucleate cells; the outline of each cell is rectangular ($5\mu \times 4\mu$), the protoplasm homogeneous, and

the nucleus rounded, with "... no marked chromatin differentiation that could be seen or with slight indications of network." In certain cases these cells occurred singly, or in groups; they are then described as being irregular or amoeboid in outline.

It will appear from the following account that there are considerable discrepancies between the observations made by Gilchrist and the author on the nature of the trophozoite, despite the fact that both have found the latter to be a uninucleate organism. It is true that the dimensions of the cells described by Gilchrist are approximately equal to those of the trophozoites of *C. thyrsites*, but the nuclear chromatin of the latter is always well differentiated. It seems likely that what Gilchrist has described as the vegetative forms are early sporoblasts, arranged in an unusual, linear order. In fixed material, the linear arrangement of erythrocytes in the blood capillaries lying upon the sarcolemma often suggests the appearance of Gilchrist's cell-chains; but the average dimensions of the erythrocyte are greater than those given by Gilchrist for the individual cells of each chain, and, in addition, he nowhere figures anything resembling a capillary wall, and the latter is nearly always apparent to assist in the discrimination of the erythrocytes. The possibility that there are some differences of vegetative habit between the Australian and South African forms of *C. thyrsites* must remain open for the present.

In the present work, all the observations made on trophozoites and spore-masses which could be allocated with certainty to *C. thyrsites* have shown that the parasite occurs within the fibres of the somatic body musculature (Plate 1, Fig. 2). In some cases, however, the parasite completely destroys the substance of the muscle fibre up to the sarcolemma and replaces it with an aggregate of trophozoites or spores. When this occurs the aggregate may appear to lie freely in the connective tissue between the muscle fibres, especially when the plane of the section is at right angles, or oblique, to the long axis of the muscle fibres. With the aid of serial sections, or of longitudinal sections of the muscle fibre, however, it can be seen that these apparently free aggregates are continuous with non-infected segments of the same muscle fibre, in which the myofibrillar substance is preserved intact.

Within the muscle fibre the parasite is usually found as a closely packed aggregate of: (a) non-sporulating trophozoites of various ages (Fig. 1), (b) sporulating trophozoites (Fig. 2), or (c) spores in various stages of maturity. These marked, phasic differences in the nature of the aggregates which infect different muscle fibres may often be observed within a small area, often as small as 1 sq. mm., of the musculature of a freshly caught, potentially "milky" fish. It may be concluded, further, that the infection is distributed throughout the host in this manner, and that the differences of phase do not correspond to any differences in the localization of the parasite within the host. The assumption that the majority of the organisms comprising each aggregate keep pace with each other, as they develop through the succession of phases described above, is supported by all the observations made during the present

investigation, subject to the qualification that, in the younger stages, a small number of organisms may be out of phase with the majority of their neighbours. These findings are in general conformity with certain observations on *Chloromyxum* (Doflein 1898) and *Leptotheca* (Kudo 1922), in which the young forms of these organisms are described as reproducing by fission before the development, at least in the case of the latter, of the sporulating phase.

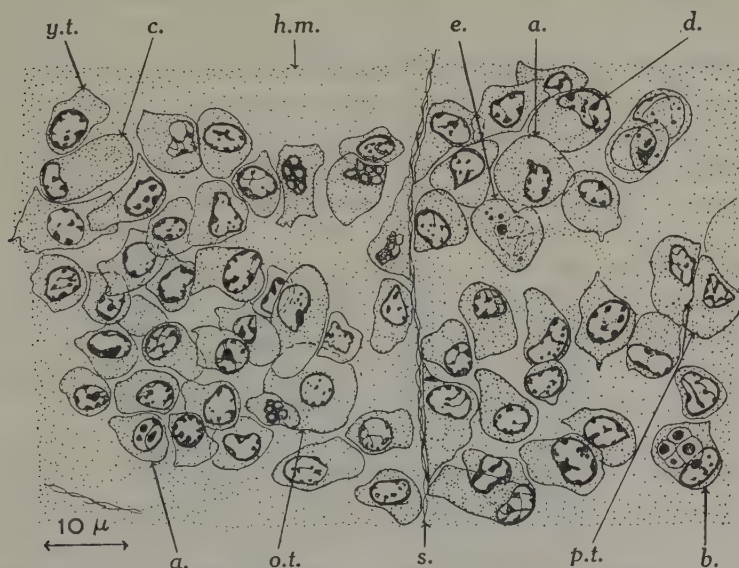


Fig. 1.—Portions of two adjacent muscle fibres infected with the trophozoites of *C. thyrstites*. From a 25 μ section of freshly fixed material. Bouin fixation, Heidenhain's iron-alum haematoxylin.

a., Trophozoites with nuclear endosomes; *b.*, trophozoites showing free cell formation or the association of two binucleate sporoblasts; *c.*, trophozoite with two sporogenous vacuoles, one with a protoplasmic inclusion; *d.*, trophozoite with elongated, folded, vegetative nucleus; *e.*, trophozoite with young sporoblast; *h.m.*, homogeneous matrix; *p.t.*, pair of closely associated trophozoites probably formed by binary fission; *s.*, connective tissue septum separating two adjacent muscle fibres; *y.t.*, young trophozoite; *o.t.*, older trophozoite.

(a) The Trophozoite before Sporulation

The appearance of an aggregate of the younger and older stages of the pre-sporulation trophozoite in two adjacent muscle fibres is illustrated in Figure 1. The young trophozoites are irregular organisms, often showing a slight elongation, but whose contours seem usually to be modified by the effects of mutual compression. The cytoplasm is homogeneous in constitution without a clearly defined cortical layer; a distinct vacuolation is often visible, even in the earliest stages.

The nucleus is almost invariably of the vesicular type, with the chromatin scattered as irregular particles upon a coarse network. In form, the nucleus is usually spherical, but may become irregular in outline. The average diameter of the spherical nucleus is 3-4 μ .

In the absence of observations on the living organism nothing can be said about the locomotory powers of *C. thyrsites*. It is possible that the fine or lobose extensions of the cytoplasm, which are occasionally to be seen, may be pseudopodial in nature. On the other hand, the mode of growth of an aggregate, which seems always to be by the peripheral extension of the original infective focus, suggests that the trophozoites lie more or less inertly within the fibre.

There is no evidence to show that the trophozoite ingests the myofibrils of the muscle fibre, or that it is in any way a holozoic feeder. The disappearance of the myofibrils before a spreading infection must be attributed either to the action of some extracellular enzyme, secreted by the trophozoites, or to some curiously localized autolysis of the muscle fibre. Aggregates of young trophozoites always appear to be immersed in a homogeneous, or sometimes granular matrix. The latter is almost certainly proteinaceous in nature, and a fluid, in the living host.

The significance of the vacuoles which appear in the cytoplasm of all stages of the trophozoite is discussed in Section IV.

It can scarcely be doubted that the aggregates of trophozoites found within the muscle fibres have been formed by the multiplication of one or a few initially infective stages. While the compact and isolated character of the aggregates, and the phasic similarities of their constituent organisms favour this view, the nature of the reproductive process remains rather obscure. All the available evidence points to the existence of a simple binary fission in the young trophozoites, preceded by binary, amitotic fission of the nucleus. In many young trophozoites the nucleus becomes elongated (Fig. 1) and often folded upon itself; in others the elongated nucleus shows a median, transverse constriction; finally, in a comparatively small number of forms (Fig. 3) the nucleus may be seen to divide, almost completely, into two spherical daughter nuclei. These stages appear to demonstrate the amitotic, binary fission of the nucleus. No division of the cytoplasm has been found to accompany the division of the nucleus and it is clear that, if cleavage occurs at all, it must be at a late stage in the division of the nucleus. The occurrence of pairs of trophozoites (as in Fig. 1, *p.t.*), in which the appearance of the nucleus and cytoplasm of each organism suggests that these have arisen by binary fission, provides the only other evidence for this mode of reproduction in *C. thyrsites*. Amitotic division of the vegetative nucleus, and binary fission of a uninucleate trophozoite have both been observed in other myxosporidians; the former occurs in a variety of genera (Davis 1923; Debaisieux 1924; Kudo 1926), the latter in *Leptotheca ohlmacheri* (Kudo 1922).

The possibility of reproduction by budding, and the question of the formation of the sporoblast nucleus are both considered in Section IV.

(b) The Sporulating Trophozoite

In the aggregates of younger trophozoites (see Fig. 1) the separate character of the constituent, uninucleate organisms can be observed directly. Later, owing to an increase in the size of the trophozoites, each aggregate forms a compact mass in which all the organisms are contiguous. When the spores are formed, the superficial appearance of the aggregate is that of a large, polysporous plasmodium (Fig. 2). Close observation, however, shows that these apparently plasmodial masses can be resolved completely into the following components: (i) spores (*sp.*), which lie in vacuoles (*v.*), enclosed by (ii) dense, but often thin, protoplasmic walls resembling those surrounding the early sporogenous vacuoles (Fig. 8), and (iii) masses of extravacuolar protoplasm (*e.p.*), which contain the vegetative nuclei (*v.n.*), and often lie excentrically at the side of the vacuoles.



Fig. 2.—Portion of a muscle fibre containing an aggregate of sporulating trophozoites. From an 18μ section. Bouin fixation, Ehrlich's haematoxylin and eosin.

e.p., Extra-vacuolar protoplasm; *s.*, sarcolemma of muscle fibre; *sp.*, spore; *v.*, sporogenous vacuole; *v.n.*, vegetative nucleus.

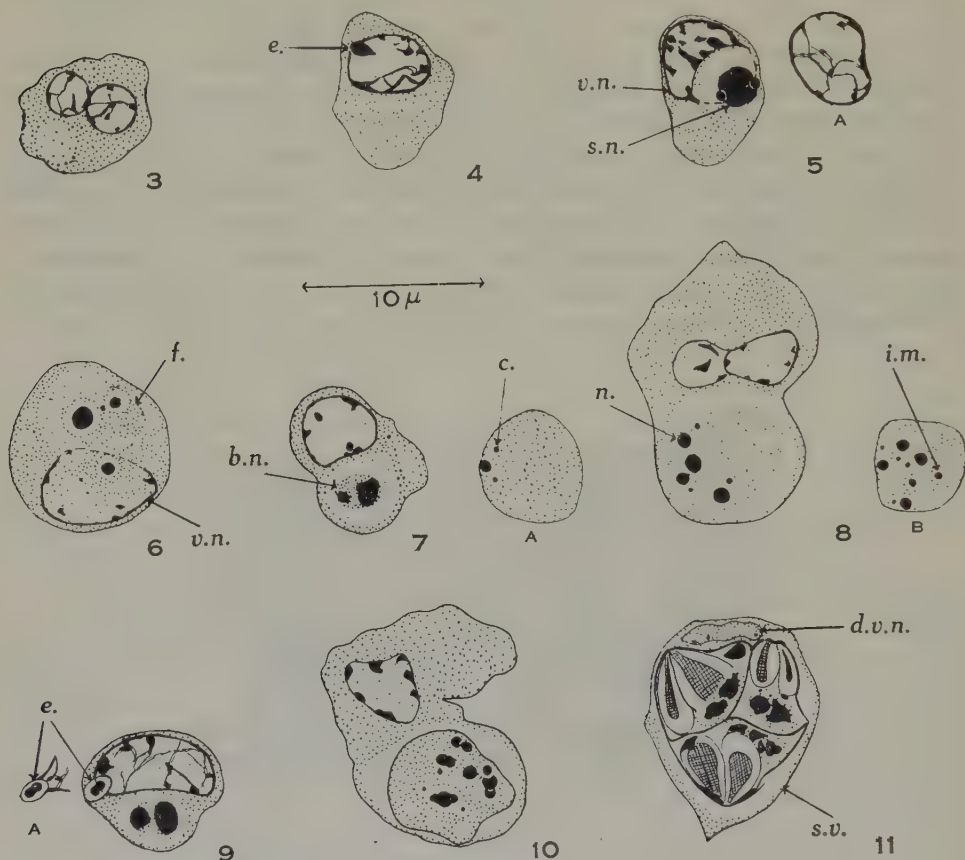
Polar capsules indicated by cross-hatching.

Since there is no distinct protoplasmic cortex around the aggregate it seems clear that no plastogamic fusion of the trophozoites has occurred, and that the aggregate is still, as in earlier stages, a mass of discrete and almost perfectly contiguous trophozoites.

Confirmation of this analysis of the sporulating aggregate is provided by post-mortem material, in which, after the breakdown of the muscle fibre, the individual sporulating trophozoites become separated.

The sporogenous vacuoles are usually elongated and interwoven in a complex manner; observations made on those which could be viewed entire showed

the presence of either one, two, or several (up to seven) spores. When several spores are present, they are often arranged in the form of a rosette (cf. Fig. 11).



Figs. 3-11.—Stages in the development and sporulation of the trophozoite. Freshly fixed material (Bouin); Heidenhain's haematoxylin. In Figure 5 the vegetative nucleus is drawn at the level of the sporoblast nucleus; 5A shows the upper level view of the vegetative nucleus. In Figure 8 the sporoblast is drawn in optical section at the middle level; A, upper level view; B, lower level view. Figure 9 shows the entire trophozoite; 9A, optical section of the nucleus at the level of the endosome.

b.n., Binucleate sporoblast; *c.*, small chromophile particle, possibly centriolar; *d.v.n.*, degenerating vegetative nucleus; *e.*, endosome; *f.*, free cell; *i.m.*, nucleus of intermediate size; *n.*, one of the large nuclei of the sporoblast; *s.n.*, sporoblast nucleus; *s.v.*, wall of sporogenous vacuole; *v.n.*, vegetative nucleus.

Compared with the nuclei of the young trophozoites, the vegetative nuclei of the sporulating forms show an increase in size and a greater irregularity of outline. The latter seems obviously to be the effect of the compression produced by the presence of the sporogenous vacuoles with their contained spores.

(c) *The Spore Aggregate*

The final phase in the development of an infective focus may be termed the spore aggregate, or "pseudocyst," to use the term introduced by Hahn (1917) in his description of the intra-muscular spore clusters formed by *Chloromyxum clupeiidae*.

The spore aggregate of *C. thyrmites* (Plate 1, Fig. 1) is the most abundant phase of the parasite to be found in the muscles of a potentially "milky" fish which has been fixed immediately after capture. In structure, it resembles an aggregate of sporulating trophozoites except that the vegetative nuclei are absent and the residual, extravacuolar masses of protoplasm are either absent or degenerate. The absence of the vegetative nucleus is apparently due to the degeneration of this structure, and not to its transformation into a sporoblast, or other form of propagative nucleus. Figure 9 shows an organism from an intermediary phase in the development of the spore aggregate, in which the vegetative nucleus is degenerating, and the chromatin only faintly basiphilic.

The walls of the sporogenous vacuoles persist in the mature spore aggregate as a delicate reticulum in which the spores are embedded. This reticulum is occasionally absent, presumably as the result of degeneration.

The forms and the dimensions of the spore aggregates vary considerably. In some cases the spores are arranged in irregular masses, either scattered throughout the homogeneous fluid within the degenerating muscle fibre, or suspended into this fluid from the sarcolemma. In other cases (as in Plate 1, Fig. 2), the infective focus appears to maintain peripheral contact with the enveloping myofibrils and to give rise to a spore aggregate with a definite, and usually ellipsoidal form. In either case, the size of the aggregate may vary from that of a small cluster of spores to that of a massive column extending almost the whole of the distance between two successive myocommata.

No cyst wall, or other defensive membrane elaborated by the host, has ever been observed around aggregates of trophozoites or spores. This is not surprising, since the periphery of the aggregates does not come into contact with the connective tissues of the host until after the post-mortem breakdown of the muscle fibres.

The variations in size shown by the spore aggregates of *C. thyrmites* are of considerable interest, since they show that the vegetative life of the trophozoites may come to an end after varying periods in the development of an infective focus. Whether the termination of trophic life is mainly determined by innate, constitutional factors in the parasite, or by changes in the immediate environment, is not known.

IV. THE SPORE AND SPORULATION

(a) *The Mature Spore*

In describing the structure of the spore (Fig. 12A-D), the surface upon which the polar capsules open will be termed the apex, and the opposite surface the base of the spore.

The spores of *C. thyrsites* are essentially pyramidal in form, with the lateral angles drawn out basally into pointed rays. The length of the spore, measured from the apex to the middle of the base, varies from 6-7 μ . The points of the rays may usually be inscribed in a square, the side of which varies in length from 12-17 μ . Sometimes, however, the rays are slightly unequal in length, one ray being longer, and its diagonally opposite fellow shorter than the two remaining rays which are usually sub-equal. These inequalities in the lengths of the rays give a dagger-shaped appearance to the spore.

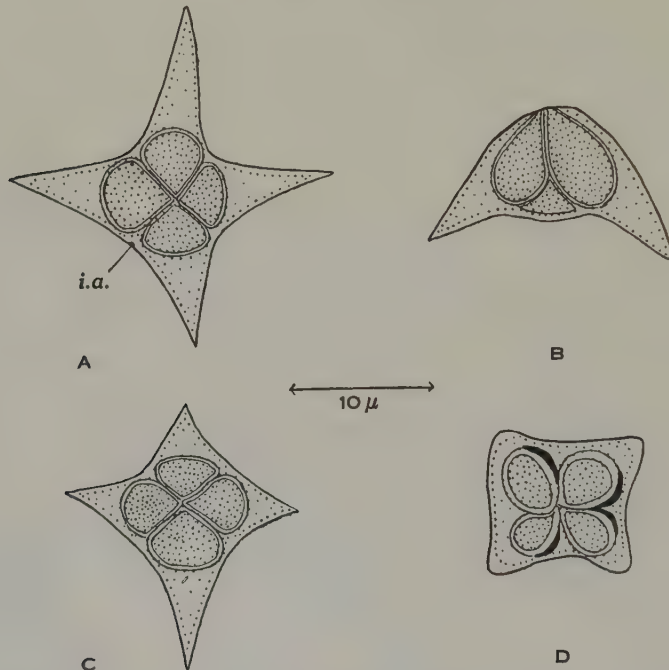


Fig. 12.—Views of the mature spore of *C. thyrsites*. A-C, Views of spores fixed and examined in formaldehyde-saline; A, viewed from the apex; B, viewed from the side; C, “dagger-shaped” spore, viewed from the apex. D, Horizontal section of spore, at the middle level, to show the characteristic arrangement of the nuclei (black) of the capsulogenous cells. Bouin fixation, Heidenhain’s iron-alum haematoxylin.

The capsulogenous cells are four in number and are lodged mainly in the apical region of the spore, but reach down unequally into the basal region which houses the sporoplasms. Each cell is ovoid, when viewed longitudinally, but circular in cross section; it consists of a thin membrane surrounding a clear space in which the elongated, essentially ovoid polar capsule is situated.

The four capsulogenous cells are invariably of unequal size. Usually, one cell is considerably larger than the others and reaches down to the base of the spore; the remaining cells may be sub-equal, or, two of them, lying diagonally opposite each other may be equal or sub-equal.

The sizes of the polar capsules vary in the same sense as their containing capsulogenous cells. Inequalities in the sizes of the polar capsules were observed by Gilchrist (1924) in the spores of the South African form of *C. thyrsites*, and also by Pérard (1928) in the spores of *C. histolyticum*.

The nuclei of the capsulogenous cells persist throughout all stages in the maturation of the spore. This is an unusual feature in the Myxosporidia, but is recorded by Kudo (1919) for *C. clupeiidae*, and also by Meglitsch (1942) for *C. opladeli*. Normally, the nuclei of the capsulogenous cells disappear in the mature spore. In *C. thyrsites*, these nuclei are rounded and vesicular in the young spore but later, and finally, the chromatin becomes condensed (pycnotic) and each nucleus takes the form of an irregular, flattened, and frequently curved disc which lies in the wall of a capsulogenous cell, along its length. Two of the nuclei occur on the adjacent surfaces of a neighbouring pair of capsulogenous cells; the two remaining nuclei occur on the opposing surfaces of their respective cells. This is a peculiarity which does not appear to have been noticed previously in *C. thyrsites*, or in any of the other species with tetradiate spores.

The substance of the polar capsules appears to be completely homogeneous and shows no trace of a spiral thread.

The sporoplasms are two rounded masses of protoplasm lying in the basal region of the spore; each possesses a single rounded nucleus. No fusion between the sporoplasms has been observed at any stage in the maturation of the spore. There is no iodophilous vacuole.

In sections of spores which have been stained with Heidenhain's iron-alum haematoxylin, a mass of intensely chromophile substance may be seen around the apices of the capsulogenous cells of the mature spore (Fig. 13C, *c.s.*). The appearance is that of slender bars which lie in the wall of the capsulogenous cell and which converge towards the apex of the spore; in the complete spore, however, the chromophile substance forms a cap around the apex of each capsulogenous cell. The significance of this chromophile material is not known.

The form of the spore, and the structural details of the spore membrane are best studied in some medium of low refractive index; water was found to be most suitable in the present studies. The spore membrane is extremely thin, and is devoid of any surface ornamentation. When the base of the spore is studied in optical section, the spore membrane is seen to consist of four parts which are separated by small, intercalated and highly refringent areas (Fig. 12, A-C, *i.a.*). As the optical focus is raised towards the apex of the spore this appearance persists for a while, but is finally confused, in the apical region, by a refraction from the apposed surfaces of the capsulogenous cells. Whether the intercalated areas of refringence in the spore membrane represent sections of two suture lines could not be decided, and the latter must be described as obscure, as in a large number of species of *Chloromyxum*. In the Myxosporidia the spore membrane is typically a bivalve structure, excepting the cases where it is described as obscure. Nevertheless, the possibility of a fundamental

quadrivalve structure in *C. thyrsites* must be borne in mind, especially in view of the close association of four nuclei with the cystogenous layer from which the spore membrane is formed in the young spore (see below).

(b) The Young Spore

The young spores (Fig. 13A and B) are spherical structures with an average diameter of 4.5μ , which is approximately equal to that of the oldest syncytial sporoblasts and also to that of the mature spore when this is measured between the bases of the rays. The fact that the diameter of the young spore is about equal to one half of that of a mature spore measured between the tips of diagonally opposite rays is accounted for by the late development of the latter.

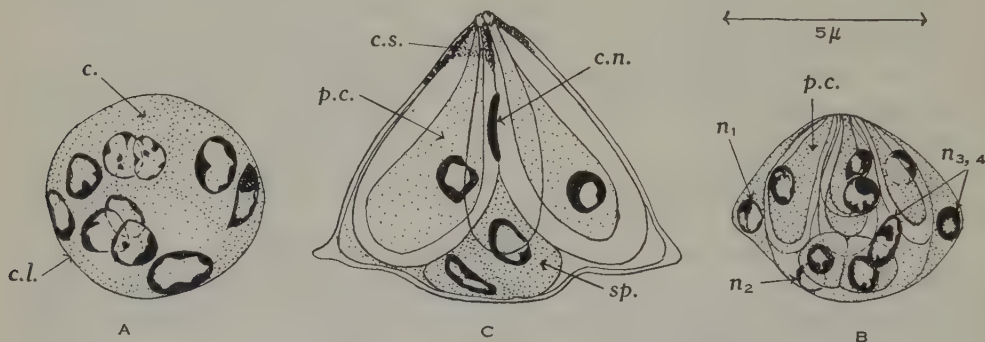


Fig. 13.—The structure of the young and mature spores. A.—Entire young spore showing the first sign of cellular differentiation. B.—Entire young spore at a later stage showing the differentiation of the capsulogenous cells. C.—Section through a mature spore. From 25μ sections of the musculature. Bouin fixation; Heidenhain's iron-alum haematoxylin. *c.*, Cell boundary; *c.s.*, chromophile substance at the apex of a capsulogenous cell; *c.l.*, cystogenous layer; *c.n.*, nucleus of capsulogenous cell; *p.c.*, polar capsule; *sp.*, sporoplasm; *n₁, n₂, n₃, n₄*, four superficial nuclei which bear a close relationship to the cystogenous layer.

The stage represented by Figure 13A is the first in which cellular organization becomes apparent in the development of the spore. The constituent cells are of varying sizes and contain a homogeneous protoplasm. Presumably, some of the inequalities in the sizes of the cells occur in the early capsulogenous cells, for the size variations of the latter are a constant characteristic of the mature spore.

Ten nuclei are present in the young spore. There are thus four nuclei in addition to those of the sporoplasms and the capsulogenous cells. It is difficult to determine the significance of the additional nuclei; they all appear to lie superficially, and at equal distances apart. Two at least lie within the superficial layer of protoplasm which covers the surface of the young spore, but it is difficult to decide whether the remaining two nuclei lie within this layer or are closely applied to it. The superficial layer of protoplasm is apparently a cystogenous or membrane-forming layer; it is thickest in the trans-

verse plane lying midway between the apex and base of the spore. In studies on other Myxosporidia it has been found that separate cells cooperate in the formation of the spore membrane. It may be suggested by comparison, that the cystogenous protoplasm of *C. thyrmites* consists of contiguous cells. No cell boundaries could be traced, however, to lend support to this view, but this may only be a reflection of the difficulties in making accurate observations on the cystogenous layer, except as it appears in profile views of the young spore.

(c) Sporulation

The establishment of the fact that ten nuclei are present in the young spore of *C. thyrmites* is of great assistance in the interpretation of the complex events occurring during the development of the sporoblast nucleus; even so, the following account is far from complete, and serves mainly to focus attention on some problems of considerable interest.

The sporoblast (Figs. 7-10) is a rounded mass of homogeneous protoplasm which is formed, and develops, in a vacuole. The latter, which may be described as the sporogenous vacuole, is surrounded by a wall of dense protoplasm. Sporogenous vacuoles of this type appear to be rare in myxosporidians; in the trophozoite of *Chloromyxum koi*, however, Fujita (1913) has described the occurrence of single spores in large spherical vacuoles lined with a layer of differentiated protoplasm.

In *C. thyrmites*, the sporogenous vacuoles appear first in the cytoplasm of the older trophozoites before the appearance of the sporoblast (Fig. 1, *o.t.*). When more than one vacuole is present the trophozoite usually assumes a spherical form which may reach a diameter of 12 μ .

It is possible that the sporogenous vacuoles may originate in the fusion of the small vacuoles which are present in the cytoplasm at all stages. On the other hand, there is a correlated increase in the average dimensions of the trophozoite which suggests that some fluid may be secreted into the vacuoles. This increase in size suggests further that the principal function of the sporogenous vacuole is to provide a space in which the spore can develop, for the mature spore may be as large as, or even larger than, the trophozoite before the formation of the vacuole; it may also protect the developing spore from the effects of compression which must occur during the muscular movements of the host.

The size of the sporogenous vacuole increases proportionately if the trophozoite becomes di- or polysporous (Fig. 11).

Although the principal features of the sporulation process are well known in many myxosporidians, there is much controversy about the origin and behaviour of the nuclei found in the early stages in the development of the spore (see Dunkerly 1925 for a review of the most important literature in this controversy). Moreover, it is beyond dispute that there are important variations, even in the outlines of the process, amongst the genera which have been investigated. Typically, a tetranucleate pansporoblast is formed at an early

stage in development; this later gives rise to two spores, as in *Myxobolus* (Keysselitz 1908); but in *Leptotheca ohlmacheri*, Kudo (1923) observed two separate, uninucleate, generative cells, each of which formed a single spore.

The typical tetranucleate pansporoblast contains a pair of large and a pair of small nuclei, and is preceded in development by a two-celled stage, with a large and a small nucleus. The association of a large and a small nucleus in the binucleate form might arise from the heteropolar division of a uninucleate cell or by the association of a small and a large free cell (microcyte and megalocyte). The former view was held by Dunkerly (1925), and the latter by Georgevitch (1914) and Keysselitz (1908). There is a similar divergence of opinion about the origin of the four-celled stage; some workers, like Dunkerly (l.c.) and Keysselitz (l.c.) believe that it is formed by the fusion of two binucleate sporoblasts, others, like Naville (1930) favour the view that the two unequal pairs of nuclei are formed by the division of the large and small nuclei of the binucleate stage.

In *C. thyrsites*, the early forms of the sporoblast (Figs. 7, 9) show an association of a large and a small nucleus which is clearly comparable to the binucleate stage of the sporoblast in *Agarella* (Dunkerly 1925) and *Myxobolus* (Keysselitz 1908) except that there is no indication of cellular organization. The chromatin of the nuclei is condensed (pycnotic) so that it appears as a homogenous mass which takes nuclear stains intensely. No evidence has been obtained to show that the binucleate sporoblast is formed by the fusion of separate free cells. On the other hand, a process of free cell formation occurs within the trophozoite; this is manifested by the appearance (Fig. 6) of small, uninucleate cells with large or small nuclei. These cells lie within a vacuole, which is presumably a sporogenous vacuole. The possibility that the binucleate sporoblast may be formed by the fusion between a large and a small free cell must therefore remain for the present. That the free cells may also represent endogenous buds, which are later set free to form uninucleate trophozoites, must also be borne in mind, especially in view of the occasional appearance, in some trophic aggregates, of small protoplasmic bodies, each with a rounded pycnotic nucleus. But against this conception of the free cell as a propagative bud must be set the observation that free cell formation occurs only in sporulating phases of the aggregates.

The question of the origin of the nuclei found in the sporoblast and the free cells remains to be considered. In *Leptotheca ohlmacheri*, Kudo (1923) observed that the generative nucleus was formed from a product of the binary fission of the vegetative nucleus of the trophozoite; this generative nucleus could be recognized subsequently because it developed certain distinctive morphological characters. That the generative or sporoblast nucleus of *C. thyrsites* may arise in a similar way is suggested by the specimen illustrated by Figure 5. In this specimen, two nuclei are present, one of which is a typical vegetative nucleus, the other, lying adjacent to it, is spherical, and shows the pycnotic character of sporoblast nuclei. Such a nucleus might give rise to the

binucleate sporoblast, either by heteropolar nuclear division, or by the formation and later fusion of free cells.

On the other hand, a large number of observations made during the present work establish the possibility that the sporoblast nuclei may be formed within the vegetative nucleus, and later extruded into the cytoplasm (see Figs. 1A, 4, 9). In the vegetative nucleus of the young trophozoite, the chromatin is scattered in the form of small irregular blocks over a coarse network; in older trophozoites, large endosomes appear within the nucleus. These are usually ovoid or spherical, and lie in contact with the nuclear membrane. Figures 4 and 9 illustrate organisms in which these endosomes appear to be extruding from the nucleus into the cytoplasm. If this interpretation is correct, it would seem that the vegetative nucleus of the trophozoite consists of somato- and idiochromatinic elements which separate at the time of sporulation.

It has proved difficult to discover the true sequence of events in the subsequent development of the sporoblast. It is clear that the sporoblast increases in size, presumably at the expense of the trophozoite, and that each nucleus divides, the daughter nuclei persisting in the pycnotic condition until the establishment of the phase which has been previously described as the young spore. Pycnosis of the sporoblast nuclei has been observed previously, for example in *Agarella* by Dunkerly (1925) who believed that it was a characteristic of the nucleus at the time of division. If this be true, then it must be that, in *C. thyrstites*, where pycnosis is shown by all the nuclei of the early sporoblast, the divisions of the nuclei follow in rapid succession, with short resting periods.

The final stages in the nuclear development of the sporoblast appear to be represented by the forms illustrated in Figures 8 and 10. In these forms there occur a large number of intensely chromophile masses of varying sizes. A careful enumeration of the particles belonging to the various size grades led to the following results:

- (i) The number of the largest particles is typically nine, but potentially ten since in one specimen (Fig. 10) one of these particles is obviously undergoing division.
- (ii) There are a variable number of small particles; in the stage illustrated by Figure 8 there are nine small particles; each of these appears to be closely related to one of the larger masses.
- (iii) In some specimens (Fig. 8) there may be particles of intermediate size, but these do not often appear.

This is admittedly a complex picture, but certain preliminary conclusions about its composition may be drawn. The first is that the larger masses are derived from the large nucleus of the binucleate sporoblast and that they represent the pycnotic precursors of the ten vesicular nuclei found in the young spore. Secondly, the distribution and behaviour of the smallest particles suggest that these are centrioles. The occasional masses of intermediate size may be derived from the small nucleus of the binucleate sporoblast; there is no evidence for their persistence beyond the stage illustrated by Figure 10.

When the trophozoite becomes di- or polysporous, each spore develops from a separate sporoblast along the lines indicated above. In such cases the development of the separate sporoblasts is usually not synchronous. There is thus no evidence for the existence, in *C. thyrsites*, of a typical myxosporidian pansporoblast such as Erdman (1916) and Woolcock (1935) described in *C. leydigi* and *C. pristiophori* respectively. This need not constitute an absolute difference between *C. thyrsites* and the other species of its present genus, however, since in the monosporocystid species of *Chloromyxum* there must presumably be atypical events in the sporulation process, but these do not appear to have received detailed attention up to the present.

The appearance presented by specimen *b* (Fig. 1) suggests that of a typical myxosporidian pansporoblast, but in view of all the other evidence it must be assumed either that free cells were being formed or that two binucleate sporoblasts have come to lie side by side.

V. SYSTEMATIC POSITION

The observations made in the present work on the vegetative phases of *C. thyrsites* make it essential to review the generic position of this species. The uninucleate condition of the trophozoite, prior to sporulation, contrasts sharply with the multinucleate plasmodium found in all the remaining species of *Chloromyxum* in which the trophozoite has been observed. This difference is sustained by the distinctive features of the spore and the sporulation process which have already been described. It would seem that the characteristics of *C. thyrsites* are sufficiently distinctive to justify its separation into a new genus. There are, however, good reasons for not taking this step at the present time. In the first place, it must be remembered that the present-day classification of the Myxosporidia is based almost entirely upon the characters of the spore, and these, as defined by Kudo (1919), justify the present generic position of *C. thyrsites*.

When the characters of the spore are used to determine the interrelationships of the existing species of *Chloromyxum*, a most interesting correlation between spore-type and parasitic habit is revealed. In all, there appear to be four recorded species of the genus in which the spore is of the quadrangular or tetraradiate type found in *C. thyrsites*; each of these species is also a parasite of muscular tissue. *C. histolyticum* Perard (Perard 1928), a parasite of the somatic muscles of the European mackerel (*Scomber scomber*), appears to bear the closest resemblance to *C. thyrsites*. It produces a quadrangular spore (12-15 μ wide) and the capsulogenous cells are of unequal size with persistent pycnotic nuclei. In addition, the presence of the parasite in large numbers is associated with a post-mortem liquefaction of the muscular tissues which closely resembles the "miliness" of barracouta which are heavily infected with *C. thyrsites*. *C. clupeiidae* Hahn (Hahn 1917) and *C. quadratum* Thélohan (Thélohan 1895) are also found as parasites of muscular tissue in a large number of fish hosts

(including *Trachurus trachurus*, the horse mackerel, in the case of *C. quadratum*.) Each possesses a quadrangular spore with polar capsules of approximately equal size. Pycnotic nuclei are persistent in the capsulogenous cells of *C. clupeiidae* (Kudo 1919). *C. funduli* Hahn (Hahn 1915), is another species which has been recorded from muscular tissues, but Kudo (1919) appears to doubt whether it is specifically distinct from *S. clupeiidae*.

It is worth noting that *C. thyrsites* and *C. histolyticum* are both restricted to scombriform fishes (*Thyrsites atun* and *Scomber scomber* respectively) and that another scombriform fish is among the hosts from which *C. quadratum* has been recorded.

It is unfortunate that, of the five species of *Chloromyxum* which produce a quadrangular spore, the trophozoite and the main features of the sporulation process are known only in *C. thyrsites*. If the distinctive features of the latter are found, by later investigations, to be shared by the remaining species, it will be necessary to create a separate genus for them, and to reserve the old genus *Chloromyxum* Mingazzini for the predominantly coelozoic forms with a plasmodial trophozoite.

VI. POST-MORTEM DEVELOPMENTS IN HOST AND PARASITE

A study of the post-mortem conditions in the host has shown that normal trophozoites may persist, although in decreasing numbers, until the onset of "miliness" in the muscular tissues. Aggregates of sporulating trophozoites have also been observed, but with certain features which distinguish them from comparable forms in the living host. Thus, the monosporous condition is predominant, and that it is final is shown by the accompanying degeneration of the vegetative nucleus of the trophozoite. Spores at all stages of maturity may be found up to 6 or 8 hours after death. After that period, and up to the onset of "miliness" the number of mature spores appears to increase. From this, and the observations made on the trophic stages, it may be inferred that the trophic life of the infection is gradually brought to an end by a final process of sporulation.

The post-mortem phases which have so far been described all conform to the pattern of the life history as it occurs in the living host. There are, however, certain anomalous forms, which are most frequently encountered about 8-12 hours after the death of the host (Plate 3, Fig. 2). Although it is not known whether these forms are sufficiently viable to produce an aggregate of mature spores, they are worth describing because it is clear that the anomalies which they show cannot all be attributed to simple post-mortem degeneration, but seem to reflect some unusual, vital response on the part of the parasite. Each of these forms consists of a compact aggregate of rounded protoplasmic masses, each with dimensions which approximate to those of a normal polysporous trophozoite. Each mass is highly vacuolated, and may contain one or several nuclei, and occasionally a small number of intensely chromophilic granules. If one nucleus is present, it may be vesicular or pycnotic,

but if several are present, they are usually all pycnotic. Plate 3, Figure 2, records a specimen in which both types of nuclei occur. The characteristic aggregates of *C. thyrsites* have been observed in all aggregates of this type, although not in large numbers. It is very difficult, at present, to interpret the significance of these post-mortem appearances. The most feasible suggestion seems to be that they represent normal trophic aggregates in which the cytoplasm of each trophozoite has increased markedly in size, possibly as the result of vacuolation, and in which sporoblast nuclei are being prepared.

After death the somatic muscular tissues of the host undergo a progressive degeneration, the flesh becoming soft and friable at first, and later, after 12-24 hours, quite fluid, with an opalescent colour.

It has already been shown that the effects of an infective focus are often very restricted. After the death of the host, however, each aggregate of trophic forms appears to exert a more extensive degenerative effect. Plate 3, Figure 1, shows the early breakdown of myofibrils in the neighbourhood of an aggregate of young trophozoites; the former break down at first into irregular blocks, and a fluid substance, which forms a granular coagulate on fixation, accumulates in the muscle fibre. That it is the various forms of trophic aggregate which produce the most extensive post-mortem damage is shown by the fact that quite thin envelopes of myofibrillae around aggregates of mature spores may persist intact for several hours after the death of the host. The visible areas of degeneration extend progressively from the infected into the uninfected areas until finally, in the "milky" fish, the entire musculature disintegrates.

The nature of the degenerative process has not been directly investigated. Pérard, who studied the association between *Chloromyxum histolyticum* and the post-mortem breakdown of the musculature in *Scomber scomber*, concluded that the degeneration was not the result of bacterial activity. Although special bacteriological methods have not been employed in the present work, it may be mentioned that there have been no signs of widespread bacterial activity in the post-mortem liquefaction of the muscles; on the other hand, close study shows that the latter is clearly correlated with the presence of *C. thyrsites*, and since, in the living host, the parasite is capable of destroying muscle fibres, without at any time appearing to feed holozoically, it seems reasonable to suppose that either the trophozoite induces autolysis of the tissues or that it secretes extra-cellular enzymes. If these suppositions are correct, it may be maintained further that the remarkable localization of the destructive effect in the living host is brought about by the removal of toxic or enzymatic substances in the blood stream. With the cessation of the circulation after death, such substances would tend to accumulate in the neighbourhood of the trophic aggregates, diffuse outwards, and produce the observed appearance of a liquefaction which proceeds progressively from the infected into the non-infected areas.

VII. ACKNOWLEDGMENTS

The author wishes to express his indebtedness to Professor O. W. Tiegs, both for his suggestion to take up the study of *C. thyrssites* and for the benefit of his experience and kindly criticism at all times. A grateful acknowledgment must also be made to Mr. M. Blackburn, Division of Fisheries, C.S.I.R.O., for his generous assistance in organizing a trip on the F.R.V. "Liawenee" for the purpose of barracouta fishing. Captain Downie (Master of the "Liawenee") and Mr. G. Scott (Technical Assistant) greatly assisted the progress of the work during the trip. For the photographs which accompany the present work, the author is indebted to the skill of Mr. Matthaei, University of Melbourne. Finally, the author wishes to express his thanks to his wife for her valued assistance in the study of the relevant literature.

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EXPLANATION OF PLATES 1-3

PLATE I

- Fig. 1.—Photomicrograph of muscle fibre infected with the trophozoites of *C. thyrssites*. 18 μ section. Bouin fixation, Heidenhain's iron-alum haematoxylin.
- Fig. 2.—Photomicrograph of a muscle fibre containing an ellipsoidal spore-aggregate of *C. thyrssites*. 10 μ section, Bouin fixation, Ehrlich's haematoxylin and eosin.

PLATE 2

Fig. 1.—Photomicrograph of degenerating muscle fibre showing sporulating trophozoites with unusually long sporogenous vacuoles. Bouin fixation, Heidenhain's iron-alum haematoxylin. 4 hours *post mortem*. 18 μ section.

Fig. 2.—Sporulating trophozoites similar to those illustrated by Figure 1 but with the sporogenous vacuoles cut transversely. 18 μ section, Bouin fixation, Heidenhain's iron-alum haematoxylin. 4 hours *post mortem*.

PLATE 3

Fig. 1.—Photomicrograph showing the histolysis of a muscle fibre in the presence of young trophozoites of *C. thyrsites*. 18 μ section. Bouin fixation, Heidenhain's iron-alum haematoxylin. 8 hours *post mortem*.

Fig. 2.—Photomicrograph showing the unusual appearance of a trophic aggregate found only in post-mortem material. 18 μ section, Bouin fixation, Heidenhain's iron-alum haematoxylin.

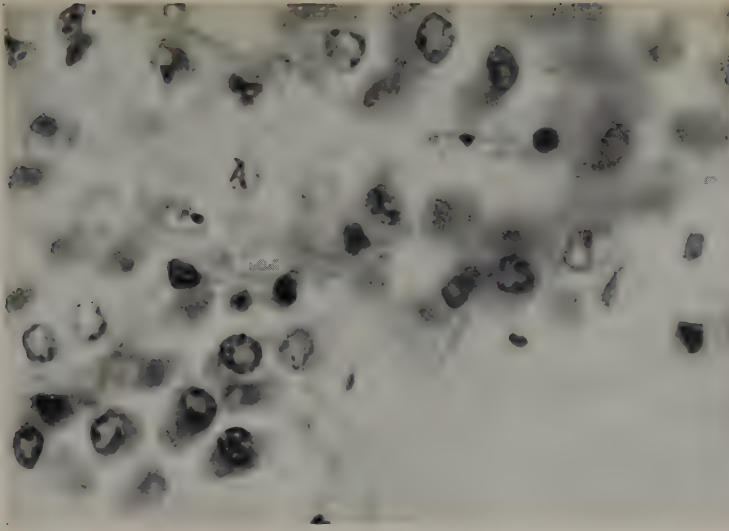


Fig. 1

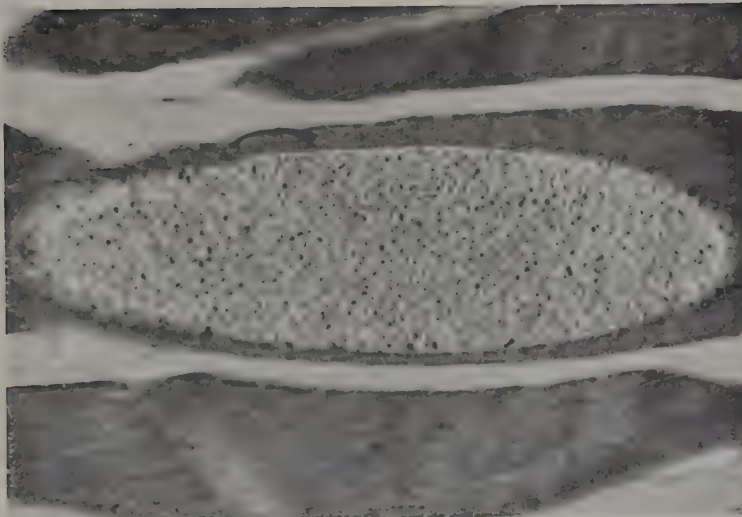


Fig. 2

WILLIS.—ON THE VEGETATIVE FORMS AND LIFE HISTORY OF *CHLOROMYXUM THYRSITES*
GILCHRIST AND ITS DOUBTFUL SYSTEMATIC POSITION

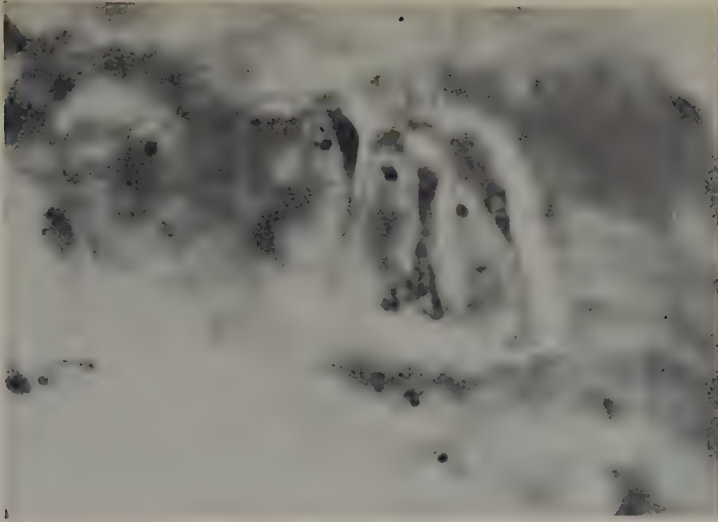


Fig. 1

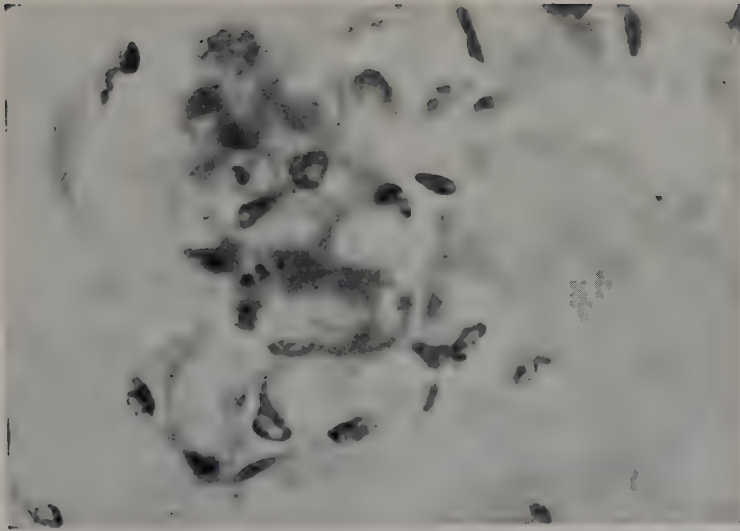


Fig. 2

WILLIS.—ON THE VEGETATIVE FORMS AND LIFE HISTORY OF CHLOROMYXUM THYRSITES
GILCHRIST AND ITS DOUBTFUL SYSTEMATIC POSITION



Fig. 1

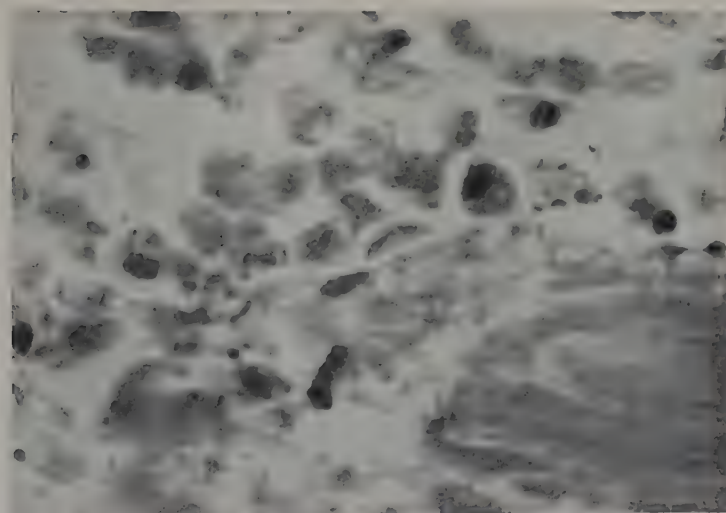


Fig. 2

WILLIS.—ON THE VEGETATIVE FORMS AND LIFE HISTORY OF CHLOROMYXUM THYRSITES
GILCHRIST AND ITS DOUBTFUL SYSTEMATIC POSITION

THE BIOLOGICAL SIGNIFICANCE OF HAEMOGLOBIN IN NEMATODE PARASITES

II. THE PROPERTIES OF THE HAEMOGLOBINS AS STUDIED IN LIVING PARASITES

By W. P. ROGERS*

[Manuscript received August 5, 1949]

Summary

In the parasites examined, the amounts of water-soluble haematin compounds, of which haemoglobin formed the large part, varied considerably; *Nippostrongylus muris* contained about 6 mg. (as haematin) per g. dry wt. of tissue, *Nematodirus* spp. and *Haemonchus contortus* about 0.8 mg. per g. dry wt. Evidence is presented which indicates that the haemoglobin of *Nippostrongylus muris* may be oxygenated *in vivo*, at least sometimes. The haemoglobin in the living parasites was easily oxygenated and deoxygenated; when the oxygen tension in the medium surrounding the parasites *in vitro* at 37°C. fell below about 13 mm. of mercury (*Nippostrongylus muris*) or 9 mm. of mercury (*Haemonchus contortus* and *Nematodirus* spp.) the oxyhaemoglobin became deoxygenated.

The rate of oxygen consumption by the three species of parasites was not significantly lessened by poisoning the haemoglobins with low concentrations of carbon monoxide at oxygen tensions between 38 and 5 mm. of mercury.

It is concluded that the haemoglobins, though present in sufficient amounts and apparently having suitable properties, are not actively concerned in the transport of oxygen in the tissues of the parasites *in vivo* when the partial pressure of the oxygen in the medium is above 5 mm. of mercury.

I. INTRODUCTION

A number of workers (for references see Rogers 1949c) have shown that haemoglobin is present in certain nematode parasites. The pigments from *Nippostrongylus muris*, *Nematodirus* spp., and *Haemonchus contortus* have properties which suggest that they may be effective oxygen carriers at the low partial pressures at which oxygen is available in the normal environments of the parasites (Rogers 1949c).

In the present studies attempts have been made to assess the physiological importance of haemoglobins as oxygen carriers in intact parasites. For this purpose, the amounts of haemoglobin in the parasites, the ability of the parasites to deoxygenate the oxyhaemoglobin in their tissues, and the partial pressure of oxygen in the medium at which deoxygenation takes place have been determined. Experiments to determine if haemoglobin of *Nippostrongylus muris* can become oxygenated *in vivo* have also been carried out. Finally, the

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effects of poisoning the parasite haemoglobins with low concentrations of carbon monoxide on the uptake of oxygen at several different partial pressures have been examined.

II. METHODS

The methods of obtaining appropriate biological materials have already been described (Rogers 1949a).

Oxygen tensions were measured electrometrically by the method of Brink and Davies (1942). The electrodes, the method of calibration, and the method of measuring currents were similar to those used by Rogers (1949c). As a rule "open" electrodes were used; as before, they were calibrated immediately before and after use.

Manometric determinations of oxygen uptake were carried out by the direct method of Warburg (1926) with small vessels of about 5 ml. capacity.

Haemoglobin was estimated, as reduced pyridine haemochromogen, by the use of a wedge trough and comparison microscope (Elliot and Keilin 1934).

III. PROCEDURE AND RESULTS

(a) *The Amount of Haemoglobin in the Parasites*

Frozen parasites were crushed and extracted several times with distilled water and the debris was separated each time by centrifuging. The combined haematin in the extract, which was largely that of oxyhaemoglobin, was then estimated as reduced pyridine haemochromogen. Though it was difficult to extract the haemoglobin from the tissues completely, this method avoided the error caused by the presence of free haematin in the intestines of some of the parasites. The total haematin found in the water extract of the three parasites is shown in Table 1.

TABLE 1
AMOUNT OF HAEMOGLOBIN FOUND IN THREE SPECIES OF PARASITES

Parasite	Haemoglobin as Haematin (mg. per 100 g. wet tissue)	
	Limits of Variation	Average*
<i>Nippostrongylus muris</i>	112-128	124
<i>Nematodirus</i> spp.	18-24	19
<i>Haemonchus contortus</i>	13-18	16

* Four separate estimations were made with each species.

The amount of haemoglobin in *Nippostrongylus muris* varied somewhat with the age of the parasite. As a rule, the parasites were recovered from the rats on the tenth day after parasitism had been established; figures for the haemoglobin content of such parasites are given in Table 1. Results obtained from the other two parasites varied much more than those from *N. muris*.

(b) *The Examination of Nippostrongylus muris Haemoglobin in vivo*

Parasitized rats which had been fasted for 6 hours were anaesthetized with "Nembutal," 40 mg. per kg., given subcutaneously. The rat was tied to a small board fitted with a clamp and so arranged that it could be placed on a microscope stage. Hair was removed from the belly of the rat and a 2 cm. incision made in the abdominal wall over the portion of the gut to be examined. About 5 cm. of small intestine containing the parasites was drawn out of the abdominal cavity and held in a clamp between two warmed glass slides. The haemoglobin in different parts of the exteriorized intestine was then examined with a spectroscope mounted in the microscope. The small clumps of worms, as seen through the intestinal wall, showed more intense oxyhaemoglobin bands than the parts of the gut where no parasites were present.

When the parasitized ether-anaesthetized rats were killed by asphyxiation with nitrogen-carbon dioxide, and the abdomen left unopened for 5-10 minutes, the parasites showed a dark colour, like that of haemoglobin, immediately after the intestine was opened. The haemoglobin became oxygenated very quickly. On the other hand, when intestines of rats were opened immediately after the animals had been killed with chloroform, the parasites showed the normal bright oxyhaemoglobin colour.

These experiments, though open to criticism, suggest that, *in vivo*, the haemoglobin of *Nippostrongylus muris* is oxygenated at certain times at least. This would be expected if the oxygen in the host gut fluids reached a partial pressure of about 15 mm. of mercury (see later in this paper). The experiments carried out by Rogers (1949c) suggest that the oxygen tensions in the contents of the small intestine of the rat may sometimes reach a partial pressure of 30 mm. of mercury.

(c) *The Deoxygenation of Oxyhaemoglobin in Intact Parasites*

Parasites were placed in a cell containing physiological saline at 37°C.; an "oxygen" electrode, a capillary bridge from a 0.15M sodium chloride half-cell, and a small coil heater were placed in position in relation to the cell, as shown in Figure 1. The heater was designed to hold the temperature of the cell and its contents between 36 and 38.5°C. The cell and heater were then placed on the axis of a microscope adjusted horizontally and from which the stage had been removed as shown in Figure 2. The arrangement was such that a beam of light could be passed through the condenser and through holes in the heater so that the contents of the cell could be examined with a low power objective and eyepiece. By this means the cell and electrodes were so adjusted that the microscope was focused on a small area just where the tip of the "oxygen" electrode was in close proximity to the parasites on the bottom of the cell. The microscope eyepiece was then replaced by a micro-reversion spectroscope. This arrangement allowed the simultaneous spectroscopic examination of the haemoglobin in the parasites and the determination of the oxygen tension in the saline adjacent to the parasites under examination.

The respiratory activity of the parasites led to the removal of oxygen from the saline in their vicinity faster than it could be replaced by the influx of oxygenated saline. As a result, the oxygen tension at the electrode tip fell, as did the current recorded by the valve microammeter. At certain oxygen

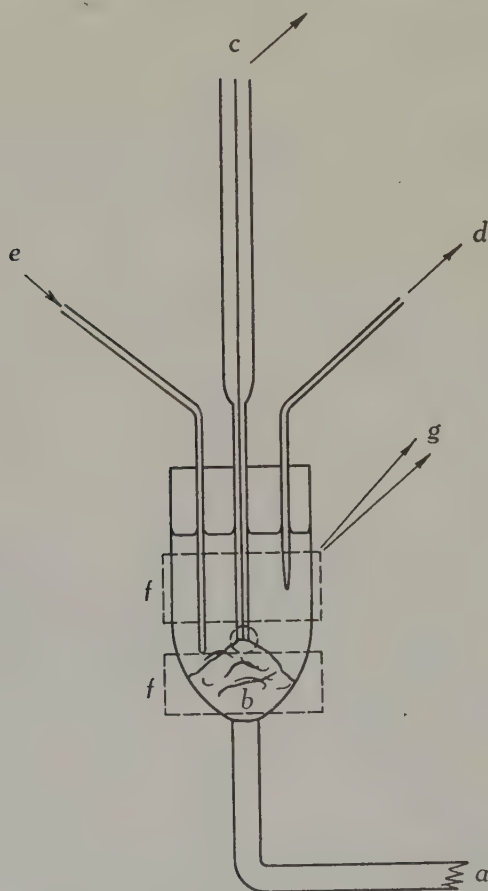


Fig. 1.—Arrangement of electrodes in the cell used for studying the deoxygenation of oxyhaemoglobin in living parasites. The cell was supported by means of the solid glass rod (*a*); the parasites (*b*) were allowed to fall to the bottom of the cell and the tip of the "oxygen" electrode (*c*) placed on the parasites in the centre of the field of the microscope (indicated by a circle). A capillary (*d*) made connection to a 0.15M NaCl calomel half-cell; when necessary, gas mixtures could be passed into the medium in the cell through the tube (*e*). The cell contents were held at a temperature of 36-38.5°C. by the perforated coil heater (*f*) connected (*g*) to a suitable power supply.

tensions, the removal of oxygen from the oxyhaemoglobin by the metabolic activities of the parasites was faster than the rate of oxygenation of haemoglobin caused by the diffusion of oxygen from the oxygen-depleted medium.

Under such conditions the oxyhaemoglobin became deoxygenated and the prominent bands of oxyhaemoglobin were replaced by the faint and diffuse band of haemoglobin. The actual fading of the oxyhaemoglobin usually took place over a period of about 30 seconds, but it was possible, by means of the reversion spectroscope, to select an approximate end-point which could be recognized without difficulty. The accuracy with which the end-points could be determined was, indeed, relatively high compared to that with which the

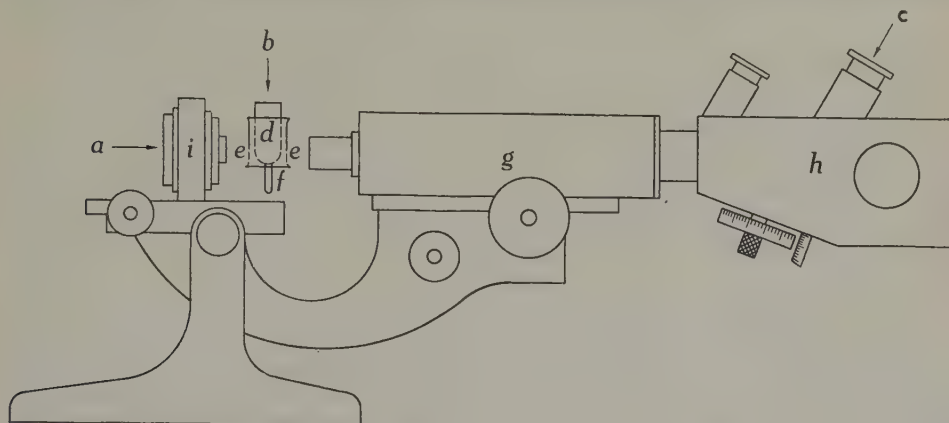


Fig. 2.—Apparatus used for studying the deoxygenation of oxyhaemoglobin in the living parasites. A strong light (a) was passed through the cell (b) and the spectrum was observed at (c). The cell, which was surrounded by a heater coil (d) perforated at (e), was supported by means of the solid glass rod (f). The micro-reversion spectroscope (h) was fixed to the barrel of the microscope (g). Light intensity was controlled by means of the condenser and diaphragm (i).

oxygen tension could be determined because the rate of fall of the oxygen tension in the medium close to the parasites was not steady. This was largely due to the active movements of the parasites themselves, which led to an irregular flow of medium past the tip of the "oxygen" electrode. The rise and fall in the electrode current was sometimes as much as 15 per cent. and only within such limits of accuracy was it possible to determine the oxygen tension in the saline, close to the parasites, at the time when the oxyhaemoglobin became deoxygenated. After a determination was made, the contents of the cell were well mixed with a stream of air; when the parasites had settled at the bottom of the cell the position of the electrode was readjusted and the determination repeated. The results obtained by examining several different lots of the three species of parasites are shown in Table 2.

(d) *The Efficiency of the Parasite Haemoglobins as Oxygen Carriers*

The proportions of carbon monoxide in all the gas mixtures shown in Table 3 were found to be sufficient to cause the formation of carboxyhaemoglobin within the parasites. These gas mixtures were therefore used to study the uptake of oxygen by parasites in which the haemoglobin was not functioning as an oxygen carrier. To decrease the possibility of carboxyhaemoglobin

dissociation during the experiments, the Warburg vessels were covered by small black bags. As a routine, at the end of each experiment in which carbon monoxide was used, the parasites were examined to make sure that the haemoglobin was largely saturated with carbon monoxide.

TABLE 2

OXYGEN TENSION (MM. OF MERCURY) IN SALINE MEDIUM, CLOSE TO THE PARASITES, WHEN THE OXYHAEMOGLOBIN WAS JUST COMPLETELY DEOXYGENATED

Parasite	Oxygen Tension (mm. of mercury)	
	Limits of Variation	Average*
<i>Nippostrongylus muris</i>	10.4-16.9	13.3
<i>Nematodirus</i> spp.	6.4-12.8	9.4
<i>Haemonchus contortus</i>	7.2-12.8	9.2

* Five determinations were made with each species of parasite.

The Warburg shaking rates were the same as those used previously (Rogers 1949*b*), which had been shown to be adequate for the type of vessel and the amount of medium used. In each experiment the oxygen uptake of the carbon monoxide-poisoned parasites was compared with that of the normal animals. The results obtained, especially at low partial pressures of oxygen, varied greatly owing to the fact that the oxygen uptake was small and the parasites tended to form clumps in the Warburg vessels at high shaking rates.

TABLE 3

PERCENTAGE INCREASE OR DECREASE IN OXYGEN UPTAKE CAUSED BY POISONING PARASITE HAEMOGLOBINS WITH CARBON MONOXIDE AT SEVERAL PARTIAL PRESSURES OF OXYGEN. EXPERIMENTS WERE CARRIED OUT AT 37°C. FOR 30 MINUTES

Gas Mixture			<i>Nippostrongylus</i> <i>muris</i>	<i>Nematodirus</i> spp.	<i>Haemonchus</i> <i>contortus</i>
O ₂	CO	N ₂			
5	2.5	92.5	Increased 4%	Increased 4%	Increased 5%
2	2	96	Increased 6%	Decreased 8%	Increased 10%
1	2	97	Increased 14%	Increased 1%	Increased 8%
0.5	2	97.5	Increased 10%	Decreased 6%	Decreased 11%

In Table 3, the effect of poisoning the parasite haemoglobins with carbon monoxide is shown as the percentage increase or decrease in oxygen uptake over that of the normal parasites, which had been examined under the same conditions except that no carbon monoxide was present. The experiments were carried out over a period of 30 minutes. No appreciable inhibition with carbon monoxide was obtained when the experiments with *Nematodirus* spp. were repeated with twice the carbon monoxide concentrations shown in Table 3.

On the whole, then, it would appear that the presence of carbon monoxide caused little change in the respiratory activity of all three species of parasites,

even at low oxygen tensions. It seems difficult, therefore, to accept the parasite haemoglobins as being effective carriers of oxygen under the conditions used in the manometric experiments. The accuracy of the method was such that small inhibitions due to the carbon monoxide might not have been detected. However, there is little doubt that if the haemoglobins were biologically important as oxygen carriers the inhibition would have been much greater, and it seems probable, therefore, that the oxygen transported to the enzyme systems of the parasites is not carried by the haemoglobins in the accepted manner.

As the failure to observe a lowered oxygen uptake in animals with carbon monoxide-poisoned haemoglobin might suggest that the respiration was limited by the availability of oxygen from the medium, in which circumstance carbon monoxide poisoning might not lower the Q_{O_2} , it is perhaps necessary to emphasize that care was taken to ensure an adequate Warburg shaking rate (see Rogers 1949a).

IV. DISCUSSION

If the haematin content of the parasite haemoglobin is similar to that of mammalian haemoglobin, it can be calculated from the oxygen consumption rates of the parasite given by Rogers (1949a), that the amount of haemoglobin in *Nippostrongylus muris* is sufficient to supply its oxygen requirements when it is respiring at its maximum *in vitro* rate, assuming that the time for half-dissociation of the oxygen from the parasite's oxyhaemoglobin (t_{50}) was less than 0.9 seconds. (The t_{50} for sheep haemoglobin is given by Hartridge and Roughton (1923) as 0.0025 second at 37°C.). With oxyhaemoglobin from *Haemonchus contortus* and *Nematodirus* spp., a t_{50} of less than 0.25 second, or less than 100 times that of the host would be required. However, at the relatively low respiration rates of the parasites *in vivo* (Rogers 1949a), the t_{50} values would have to be at least three times those given above before the turnover rate would begin to limit respiration if all the oxygen was transported via oxyhaemoglobin. It was obvious, without detailed examination, that the haemoglobins of the Trichostrongyle parasites had t_{50} values very much less than those of the perienteric fluid of *Ascaris lumbricoides*, 1000 ± 100 seconds, or its body wall, 250 seconds (Davenport 1945), though it could not be ascertained without elaborate experiment what the actual t_{50} values were. As it was found that carbon monoxide poisoning did not reduce the oxygen uptake of the parasites, even at lowered oxygen tensions, when respiration was slow, it seems unlikely that the failure of the haemoglobin to transport physiologically significant amounts of oxygen was due to the slow dissociation of the oxyhaemoglobin.

The validity of the experiments, which were carried out to determine the condition of the haemoglobin in *Nippostrongylus muris* in the intact host intestine, depended among other things on whether the amount of haemoglobin in the parasites was larger than that in the host tissue. Porter (1935) stated that the bright red patches found in the intestines of parasitized host animals were due to excess blood in the villi rather than to the pigment in the parasites

themselves. Though there is no doubt that the villi were dilated where the "worm nests" were situated, the high concentration of oxyhaemoglobin in the parasite tissues was such that, in the present experiments, microscopic examination showed the blurred red tissues of the parasites through the walls of the host intestine. It was therefore possible that the increased density of the oxyhaemoglobin bands where the parasites were situated was due to the parasite haemoglobin. Unfortunately, the general absorption due to the walls of the host intestine made it very unlikely that the faint diffuse band of haemoglobin would have been detected if the pigment in the parasites had been deoxygenated. However, the finding of the bright red colour in the parasites when they were examined immediately after the death of the host, whereas the darker colour of the haemoglobin was present when the host intestine was not opened until some 5 minutes after death by asphyxiation, gave further support to the indication that *Nippostrongylus muris* haemoglobin was oxygenated *in vivo*.

Owing to the thickness of the walls of the sheep intestine and abomasum, and to the low concentration of haemoglobin in *Nematodirus* spp. and *Haemonchus contortus*, the examination of the haemoglobin in these parasites could not be carried out in the live host.

The oxygen tensions at which the oxyhaemoglobin in the living parasites was deoxygenated were much higher than those found when purified oxyhaemoglobin solutions were used (Rogers 1949*b*). This may have been due to the fact that, in the *in vivo* studies, the oxygen tension measured was that of the medium close to the parasites and not that of the actual tissue containing the haemoglobin. In the medium, even close to the parasites, the oxygen tension may well have been much higher than that in the tissues. Further, the oxyhaemoglobin in the tissues of *Nippostrongylus muris* reached a much higher concentration than that used in the solutions, which would have caused an increase in the affinity for oxygen (Hill and Wolverkamp 1936). Other factors which may have been concerned were temperature (16°C. was the temperature used in experiments with the purified pigment), pH, and salt concentration. However, it is clear that, in order to maintain fully oxygenated haemoglobin in the parasites, the partial pressure of oxygen in the host gut fluids, in which the parasites normally live, would have to reach 13 mm. of mercury for *Nippostrongylus muris* and 9 mm. for the sheep parasites. Though oxygen tensions of the order of 13 mm. of mercury probably occur in the contents of the small intestine of the rat close to the mucosa, it is doubtful if pressures of 9 mm. are reached in the contents of the sheep's abomasum or in that part of its small intestine where *Nematodirus* spp. normally live (Rogers 1949*c*).

Many invertebrates, e.g. *Tubifex tubifex*, *Sabella pavonina*, and *Lumbricus herculeus*, have haemoglobins which have been found to be efficient oxygen carriers at atmospheric pressures (Dausend 1931; Ewer and Fox 1940; Johnson 1942). The haemoglobins of some Chironomid larvae, however, do not transport physiologically significant amounts of oxygen when the surrounding

medium is more than 25 per cent. (*Tanytarsus*) or 44 per cent. (*Chironomus*) saturated with air (Walshe 1947; Ewer 1942). The haemoglobin in *Daphnia*, like that in Trichostrongyle parasites, is not functionally active as an oxygen carrier even at low partial pressures of oxygen (Fox 1948).

Laser (1944) has shown that the nematode *Ascaris lumbricoides* lacks catalase; when this parasite is exposed to high concentrations of oxygen, hydrogen peroxide accumulates in the tissues in toxic amounts. If catalase is generally absent from the tissues of nematode parasites, the possibility that the haemoglobins may have some physiological value as peroxidases might be considered.

V. ACKNOWLEDGMENTS

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THE BIOCHEMICAL CHARACTERIZATION OF A SEROLOGICALLY ACTIVE LIPID FRACTION OF THE NEMATODE *HAEMONCHUS CONTORTUS*

By A. C. JENNINGS*

[Manuscript received August 12, 1949]

Summary

Protein, polysaccharide, and lipid fractions were prepared from *Haemonchus contortus*. The lipid fraction appeared to be the only one essentially concerned in reactions with natural antisera from sheep, and further work was carried out to determine the nature of the serologically active lipid. It was found to be hydrophilic, acidic, susceptible to oxidation, free from protein and polysaccharide, and to have a molecular weight greater than 1000. Its biochemical significance, rôle in the activity of the boiled antigen, and possible relation to other lipoidal antigens are discussed.

I. INTRODUCTION

Stoll (1932) showed that sheep could develop a pronounced resistance to infestation with *Haemonchus contortus* and this has been confirmed and extended by Gordon (1948). Earlier workers (see Stumberg 1933) had commented on the presence or absence of antibodies to *Haemonchus contortus* in sheep sera, and Hawkins and Cole (1945) had found that when exsheathed strongyle larvae were placed in sera from resistant sheep, a precipitate formed at various sites on the larval surface; however, Stewart (1948) was the first to demonstrate clearly by classical methods the presence of antibodies against *Haemonchus contortus* and *Trichostrongylus* spp. in sheep sera and to study their rôle in the resistance of sheep to these parasites.

The immunochemical studies here described had as their objective the identification of the biochemical fraction of the parasites responsible for the serological reaction.

The polysaccharide fractions of helminths have been examined by several workers, especially by Campbell (1936, 1937, 1939) and Gonzalez (1943, 1946), who showed that helminth polysaccharides have many of the characteristics of bacterial polysaccharides. Others (Brisou 1946; Culbertson, Rose, and Gonzalez 1947; Warren 1947) have studied the serological activity of aqueous extracts of helminths. Apart from the work of Fairley (1925, 1927), Kellaway (1928), Wharton (1930), and earlier workers, on the lipids of helminths, little is known of their nature, specificity, and importance in immunology. These workers found that alcoholic extracts of the helminths reacted with

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corresponding antisera; Fairley and Kellaway found that these lipids could function as antigens, whereas Wharton considered they could act only as haptens and also noted that the specificity of the lipids which he prepared was low.

In this Laboratory, protein, polysaccharide, and lipid fractions were prepared from *Haemonchus contortus*. As only the lipid fraction showed pronounced serological activity, it was the only fraction investigated in detail. The serological activity of the various fractions was titrated by means of a complement fixation reaction and the author is indebted to his colleague, Mr. D. F. Stewart, for carrying out these tests.

II. MATERIALS AND METHODS

(a) Collection of Material

The collection and treatment of biological materials in the fresh state was achieved by the following techniques, designed for the rapid collection of large amounts of material.

A method devised by Mr. H. V. Whitlock of this Laboratory was employed for collecting and cleaning nematode eggs. It may be described briefly as follows:

A large quantity (up to 2 or 3 kg.) of freshly passed faeces containing a large number of ova is thoroughly broken down with pestle and mortar and then mixed with a quantity of water to make a free-flowing suspension. This is allowed to stand overnight in a refrigerator. The suspension is then poured into a fine mesh sieve (approximately 30 meshes per inch) and washed several times to obtain the maximum number of ova free from the larger pieces of debris. It is then mixed with an equal volume of sugar solution (150 g. sugar in 100 ml. water). Large shallow enamel trays 14 by 10½ by 2 in. are fitted with glass plates cut so as just to fit inside the tray, with one corner cut off to permit the introduction of rubber tubing, and with handles cemented to the upper surface at each end so as to overlap the ends by 1 in. and to serve as supports. The glass plate in position lies just below the level of the rim of the tray. The glass plate is placed in position. The suspension is poured into the tray until it makes contact with the entire surface of the glass plate. The preparation is allowed to stand for approximately 30 minutes, when the ova will have floated to the surface and will be adhering to the under surface of the glass plate. It is essential that the glass plate is thoroughly dry and clean before use.

A length of rubber tubing is introduced through the opening at one corner of the glass plate and portion of the faeces-sugar suspension siphoned off. The plate with an adhering film of fluid containing large numbers of ova may then be lifted from the tray. The ova are then washed off and the washings transferred to a narrow container to sediment, or alternatively they may be centrifuged. The ova should be washed with water several times. Concentration between washings is achieved either by sedimentation or centrifugation.

The flotation process may be repeated several times on the material in the tray; further quantities of ova will thereby be obtained. At each repetition the glass plate is carefully washed and dried before use.

Infective larvae from faecal cultures were cleaned and washed in distilled water by a modification of a technique described by Hamilton (1950) for the collection of *Trichostrongylus* spp., a No. 2 sintered-glass filter being used instead of metal gauze filters. After the larvae had been washed off the filter, concentration was effected by centrifuging.

Small species of nematodes such as *Haemonchus contortus* and *Trichostrongylus* spp. were collected by the method described by Hamilton (l.c.) and then dried between filter papers. Larger helminths, such as *Moniezia expansa* and *Fasciola hepatica*, were merely washed in saline and dried with filter paper before use.

(b) Treatment of Material

It was found that the cleaned nematode eggs and larvae could be macerated satisfactorily in a conical, ground-glass bacterial mill without the use of powdered glass. Adult nematodes and animal tissues were ground in a mortar and pestle, without the addition of sand.

In some experiments an aqueous suspension of macerated material or a boiled antigen preparation were lyophilised before extraction with the appropriate solvent. Lyophilisation was effected by the technique of Flosdorf and Mudd (1935).

Conditions favouring the rancidification of fats caused deterioration of the serological activity of the lipid fractions, hence all such material was stored and treated under nitrogen and kept in the cold as much as possible.

(c) Preparation of Fractions

Lipid fractions were prepared from helminths and animal tissues by extraction with absolute alcohol. *Haemonchus contortus* was extracted with other solvents in order to study the nature and solubility of the serologically active lipid. After the first experiments, a standard empirical method was used for the preparation of all lipid fractions.

One part of the material to be extracted was macerated and transferred to a gas-tight, ground-glass stoppered Erlenmeyer flask together with one hundred parts of the appropriate solvent. "Quickfit" apparatus was always used to avoid possible contamination from cork or rubber. The contents of the flask were thoroughly gassed with nitrogen and the extraction was then left in the refrigerator at 4°C. The actual time of extraction ranged from 24 hours to two weeks in different experiments without any apparent effect on the potency of the final preparation.

The extraction was centrifuged and the supernatant filtered through filter paper to remove any remaining particulate matter. The solvent was removed by vacuum distillation under nitrogen. With absolute alcohol as solvent, the

temperature of distillation was usually of the order of 25°C. Temperatures of distillation above 30°C. caused a deterioration of serological activity of the lipid fractions.

After removal of the solvent, the brownish-white greasy lipid residue was suspended in 25 parts of 0.9 per cent. sodium chloride solution and stored under nitrogen in the cold. The lipid suspension so formed was white, opaque, and physically stable, at no time showing any tendency to precipitate, and it was used in this form for serological tests. For extraction, 100 parts of solvent to one part of fresh material were used to keep the water content as low as possible. The lipid residue from one part of material was suspended in 25 parts of physiological saline so that the results in the serological tests would be directly comparable with those obtained with the boiled antigen used by Stewart (1948).

All solvents used in this work were the purest obtainable and all were redistilled before use. The redistilled anaesthetic ether was stored in the dark over sodium.

Polysaccharides were prepared from *Haemonchus contortus*, *Nematodirus*, *Ascaris lumbricoides*, *Moniezia expansa*, and *Fasciola hepatica* by the method of Melcher and Campbell (1942), followed by treatment with chloroform (Sevag, Lackman, and Smolens 1938), until free from protein and negative to the biuret test.

In some experiments protein solutions were prepared from saline extracts of *Haemonchus contortus*. An equal volume of saturated ammonium sulphate solution was added to the saline extracts, then 10 per cent. trichloroacetic acid to the point of maximum precipitation. The precipitate was collected and dialysed against saline till free from sulphate and trichloroacetate ions.

Thorough absolute alcohol extractions of various helminths was followed by saline extraction of the alcohol-insoluble residue in another series of experiments, the saline-soluble extracts being used as protein fractions without further treatment.

Nematodes were also extracted under nitrogen by concentrations of aqueous alcohol ranging up to 50 per cent. alcohol, followed by treatment with ammonium sulphate and trichloroacetic acid, as above, and dialysis under nitrogen of the supernatants and precipitates against 5 per cent. alcohol in distilled water till free of sulphate. After centrifuging, both supernatants and precipitates were used as protein fractions. The alcohol content did not interfere in the complement fixation test. In all experiments solutions were restored to isotonicity before testing or injection.

III. RESULTS

(a) Properties of the Lipid Fraction

(i) *Extraction and Solubility of the Lipid.*—In the first stages of this work the serologically active lipid of *Haemonchus contortus* was found to be soluble in absolute alcohol. *Haemonchus contortus* was then extracted with other

solvents to determine the solubility and nature of the active material. The extracts were tested as saline suspensions for serological activity. The activity of the extracts is shown in Table 1.

TABLE 1
SEROLOGICAL ACTIVITY OF MATERIAL EXTRACTED BY SEVERAL SOLVENTS
FROM FRESH *HAEMONCHUS CONTORTUS*

Solvent	Reaction of Extract with <i>Haemonchus contortus</i> Antiserum
Absolute Alcohol	+++++
Alcohol 75%	+++++
Alcohol 50%	+++++
Methyl alcohol	+++++
Propyl alcohol	—
Butyl alcohol	+
Amyl alcohol	—
Anaesthetic ether	++
Petroleum ether (60-80°C. fraction)	+
Benzene	+
Carbon tetrachloride	±
Chloroform	—
Acetone	—
Phenol 2%	+++
Pyridine	+++++
Diethylene glycol	+++++
Absolute alcohol (10N hydrochloric acid to pH 3.0)	—
Absolute alcohol (40% sodium hydroxide solution to pH 10.0)	—
Absolute alcohol 100 ml. + aqueous trichloroacetic acid 30 ml. at pH 3.0	+++++
Aqueous N/4 trichloroacetic acid	—

++++ indicates high activity; — indicates no activity.

Certain of the organic solvents caused the macerated worm material to coalesce into gummy lumps and this possibly prevented complete extraction. To overcome this, the macerated worm material was lyophilised before extraction by a selection of these solvents (see Table 2). This method extracted

more material but without much change in serological activity. The activity of these extracts is shown in Table 2.

TABLE 2
SEROLOGICAL ACTIVITY OF MATERIAL EXTRACTED BY SEVERAL SOLVENTS
FROM LYOPHILISED *HAEMONCHUS CONTORTUS*

Solvent	Reaction of Extract with <i>Haemonchus contortus</i> Antiserum
Absolute alcohol	+++++
Anaesthetic ether	+
Benzene	±
Chloroform	±

+++++ indicates high activity; ± indicates only a trace of activity.

Ether and petroleum ether (60-80°C. fraction) extracted the active material from acidified aqueous suspensions of the lipid, but not from neutral or alkaline suspensions. On the other hand, chloroform extracted the active material from aqueous suspensions at any pH. The serologically active lipid, together with other material, was precipitated from aqueous suspensions on acidification below pH 2.5. Further details of these points are given later in this article.

(ii) *Stability of the Lipid*.—As already noted, the active lipid of *Haemonchus contortus* was unstable under conditions favouring rancidification of fats. This rendered chemical work very difficult and imposed rigorous limits on experiments designed to identify the serologically active lipid, because detection depended on serological activity. Saline suspensions of the lipid of *Haemonchus contortus* only retained their serological activity for two to three weeks in nitrogen in the cold and attempts were therefore made to stabilize it with antioxidants.

Pyrogallol, added to the saline suspensions in concentrations of 0.01 per cent. and 0.005 per cent., and hydroquinone added in a concentration of 0.01 per cent., did not appear to confer stability. However, this approach was abandoned because the antioxidant caused so much darkening of the erythrocytes used in the complement fixation tests that the results could not be read with any degree of accuracy.

The addition of Raney-nickel in a concentration of 0.1 per cent. during extraction with absolute alcohol, and gassing with hydrogen instead of nitrogen, did not enhance the activity of the saline suspension of the lipid when compared with a control extraction.

At first it was thought that exsheathing of the *Haemonchus contortus* larvae would result in better maceration, but the exsheathing was found unnecessary. Incomplete washing after treatment with sodium hypochlorite also gave preparations of the lipid fraction and of boiled antigen which were unsatisfactory for serological work.

(b) Quantitative Aspects of the Lipid Fraction

In the few experiments with *Haemonchus contortus* eggs, absolute alcohol gave extracts of much higher serological activity than those obtained from third-stage larvae or adults. The third-stage larvae consistently yielded lipid of high activity, whereas the activity of lipid obtained similarly from adults was very variable.

Fresh batches of *Haemonchus contortus* larvae were collected and divided into two equal lots. One lot was immediately macerated and extracted with absolute alcohol as above; the other lot was allowed to age in an incubator at 30°C. for one week, until most of the larvae were very sluggish, before maceration and extraction with absolute alcohol. The saline suspensions of lipid obtained from these extracts were equally active.

Approximately 0.015 g. of material could be extracted by absolute alcohol from 1 g. of centrifuged *Haemonchus contortus* larvae. If the larvae were first treated with acetone, or were allowed to age, the quantity of material extracted by absolute alcohol was much reduced although its serological activity was not diminished. It follows, therefore, that the serologically active lipid was present only in small amounts in the absolute alcohol-soluble material.

(c) Cross Reactions of Helminth Lipids

Saline suspensions were also prepared of the absolute alcohol-soluble lipids of other helminths and of some vertebrate tissues, and these were tested for serological activity against sheep sera known to contain complement-fixing antibodies only for *Haemonchus contortus* antigen. The immunological significance of the cross reactions obtained will be discussed elsewhere by my colleague, Mr. D. F. Stewart; the results are summarized in Table 3, merely as an indication of the distribution of the lipid among helminths.

(d) Nature of the Lipid from *Haemonchus contortus*

The first fractionation of the alcohol-soluble material of *Haemonchus contortus* was made by extracting aqueous lipid suspensions at different pH levels with ether or petroleum ether (60-80°C. fraction). The fraction obtained by extraction of the alkaline lipid suspension was known as the "unsaponified" fraction. The ether extract obtained after acidification of the suspension to pH 2.0, was called the "fatty acid" fraction.

Both ether extracts, after washing with distilled water, were evaporated to dryness by vacuum distillation under nitrogen. Saline suspensions of the lipid fractions were then prepared as above; when tested by the complement-fixation test the "unsaponified" fraction was inactive, whereas the "fatty acid" fraction was quite active.

The pH of aqueous suspensions of the lipid was usually found to be about 5.0, i.e. slightly more acid than the distilled water used (pH 5.5 to pH 6.0). Ether extraction at this pH did not remove any active material, but on acidifica-

tion to pH 2.0, active material was obtained as before. On the other hand, active material could be extracted by chloroform from aqueous suspensions of lipid at alkaline, neutral, or acid pH.

TABLE 3
CROSS REACTION OF LIPIDS FROM DIFFERENT HELMINTHS
WITH *HAEMONCHUS CONTORTUS* ANTISERUM

Material Extracted	Reaction with <i>Haemonchus contortus</i> Antiserum
<i>Haemonchus contortus</i> } adults	+
} larvae	+
} eggs	+
<i>Nematodirus</i> —adults	+
<i>Trichostrongylus</i> } adults	?
} larvae	+
<i>Oesophagostomum</i> —adults	+
<i>Moniezia expansa</i> —adults	+
<i>Ascaris lumbricoides</i> —adults	+
<i>Chabertia ovina</i> —adults	+
<i>Dictyocaulus filaris</i> —adults	+
<i>Strongyloides</i> —larvae	+
<i>Fasciola hepatica</i> —adults	—
<i>Paramphistomum cervi</i> —adults	—
Beef heart	—
Beef liver	—
Sheep's brain	—
Sheep's intestinal mucosa	—
Hen's egg yolk	—

The addition of hydrochloric acid or sulphuric acid to pH 2.0 in these experiments, followed by warming in nitrogen, resulted in the formation of greasy globules which floated on the surface and dissolved readily in ether. Treatment of aqueous suspensions of the alcohol-soluble lipid with trichloroacetic acid in the cold to a pH less than 2.5 gave a flocculent precipitate containing the active lipid, which could be readily centrifuged off and resuspended in saline. If the solution was made alkaline after acidification with trichloroacetic acid, the precipitate redissolved to give the characteristic opaque lipid suspension.

The alcohol-soluble material of *Haemonchus contortus* was also subjected to hydrolysis. Saturated solutions of barium hydroxide, pig pancreatic lipase, and alcoholic solutions of potassium hydroxide were used as hydrolytic agents, all experiments being carried out in nitrogen. No significant results could be obtained, since the loss of serological activity was so great in the control experiments, from which the hydrolytic agents were omitted, that the inactivation in the presence of the hydrolytic agents could not be attributed to hydrolysis alone.

A molecular still was used in a further attempt at fractionation. The alcohol-soluble material of *Haemonchus contortus*, after degassing by evacuation of the still without external heating, was subjected to distillation at pressures of the order of 0.01 mm. mercury and at temperatures ranging up to 290°C. The distillates were white or very light brown in colour and, when suspended in saline, were always serologically inactive. The residues showed considerable decomposition, being dark red or black in appearance; after suspension in saline and light centrifugation to remove particulate matter, the light brown opaque supernatants showed serological activity. The stability of the active material under these conditions was probably due to the absence of moisture and oxygen.

The material extracted by absolute alcohol from *Haemonchus contortus* was negative to Molisch and "rapid furfural" tests and negative to colour tests for proteins, although some samples gave a doubtful positive biuret test. One sample of material was analysed and found to contain 0.06 per cent. total P, 2.0 per cent. total N, and 1.4 per cent. reducing substances (calculated as glucose) after hydrolysis. These figures have little significance as far as the serologically active lipid is concerned but show that the phospholipid content of the absolute alcohol extract was low and that nitrogenous compounds and possibly carbohydrates were present in the extract.

The active lipid of *Haemonchus contortus* would not dialyse through cellophane tubing and this fact was used in experiments to remove salts formed during neutralization, which otherwise would affect the isotonicity of the saline suspensions of the lipid fractions subsequently prepared. Dialysis was carried out under nitrogen against distilled water.

(e) *The Lipid in Relation to the Boiled Antigen*

The close similarity in serological activity between the absolute alcohol extract and the boiled antigen prepared from *Haemonchus contortus* suggested that the active lipid might be concerned in the serological activity of the boiled antigen.

It was known that saline extracts of *Haemonchus contortus* had only slight activity in the complement fixation test, and that saline extracts of *Haemonchus contortus*, previously extracted with absolute alcohol, were without activity. It was found necessary to extract 1 g. lots of *Haemonchus contortus* with four 100 ml. lots of absolute alcohol to remove all serologically active lipid. A boiled antigen prepared from the extracted residue of *Haemonchus contortus* was inactive.

To determine whether the serologically active lipid was present in the boiled antigen and, if so, in what form, a boiled antigen was prepared, but was not made isotonic, and was then lyophilised. It was then extracted with absolute alcohol and the alcoholic extract treated as above. The resultant saline suspension of the lipid was found to be active. It was necessary to extract the lyophilised boiled antigen with at least three 100 ml. lots of absolute alcohol to remove all the extractable active material. The residue was then resuspended in 25 ml. saline and it too, was found to be serologically active.

(f) Protein and Polysaccharide Fractions

Stewart (personal communication) found that the protein solutions and the polysaccharide fraction of *Haemonchus contortus* would not fix complement with antisera from naturally infested sheep, and for that reason their chemical nature has not been examined.

IV. DISCUSSION

The organic solvents which extracted serologically active material from *Haemonchus contortus* with any degree of efficiency, were miscible with water and the majority were protein denaturants and contained hydroxyl groups. It is possible that a presumably physical combination of lipid with protein must be broken down as a preliminary step in the extraction; this would explain why only slight activity was found in saline extracts of macerated material, especially if a saline-insoluble structural protein were involved.

This is supported by the fact that, although repeated absolute alcohol extraction of *Haemonchus contortus* removed all serologically active lipid, this was not so with the boiled antigen, suggesting that the serologically active lipid entered into varying degrees of physical, rather than chemical, combination with absolute alcohol-insoluble protein in the boiled antigen. Also, the boiled antigen did not have to be stored under nitrogen (Stewart 1948) which meant that the serologically active lipid was stabilized.

That the serologically active lipid of *Haemonchus contortus* was hydrophilic and acidic was shown by the nature of the solvents which extracted it from *Haemonchus contortus*; by the formation of very stable aqueous suspensions; by the precipitation of the serologically active lipid, together with other acid-precipitable material, on acidification of aqueous suspensions below pH 2.5; by its extraction with ether or petroleum ether from acidified aqueous suspensions but not from alkaline or neutral aqueous suspensions. The hydrophilic and acidic properties of the serologically active lipid would facilitate the formation of physical aggregates with protein.

The instability of the serologically active lipid of *Haemonchus contortus* under conditions favouring rancidification rather than disintegration suggests that the lipid was unsaturated and easily oxidized but was not an unstable complex aggregate involving two or more compounds. This suggestion is supported by its stability during molecular distillation. The molecular size of

the lipid was indicated by its failure to distil in the molecular still and its inability to dialyse through cellophane. Fawcett (1939, 1948) points out that the upper molecular weight limit for practical molecular distillation of organic substances is in the region of 1000 and that organic substances with higher molecular weights generally undergo thermal decomposition before the vapour pressure necessary for distillation is achieved; phosphatides and proteins decompose and cannot be distilled. Apparently, then, the serologically active lipid has a molecular weight greater than 1000, and a relatively stable molecular configuration, since it did not readily undergo disintegration.

The absence of polysaccharide or protein in the absolute alcohol-soluble serologically active material of *Haemonchus contortus* was indicated by the negative protein and polysaccharide colour tests, the ease of solution in ether and petroleum ether after acidification of aqueous suspensions of the absolute alcohol-soluble material, and the behaviour of the active lipid in the molecular still. However, the presence of very small amounts of nitrogenous compounds and simple saccharides in the serologically active lipid cannot be excluded.

To summarize these points briefly, the serologically active lipid probably occurs in *Haemonchus contortus* in loose physical combination with protein, it is hydrophilic, acidic, and susceptible to oxidation, with a molecular weight probably greater than 1000 and is presumably free from protein and polysaccharide; it apparently combines physically with protein in the boiled antigen, in which the serological activity of the lipid is stabilized.

Properties of the Boivin, Forssman, and Brucella (Miles and Pirie 1939; Paterson, Pirie, and Stableforth 1947) antigens which are similar to those of the serologically active lipid may be given as follows:

The Boivin antigen from *Salmonella typhimurium* is extracted by diethylene glycol from dried cells, is non-dialysable, forms opalescent solutions, and is very unstable. The Brucella antigen (Paterson, Pirie, and Stableforth 1947) is readily precipitated from crude solutions with other acid-precipitable material, is extracted from the organisms by 2 per cent. phenol, dissolves in pyridine with disaggregation, and the antigen is disaggregated and part thereof extracted by chloroform from acetate buffer at pH 4.0. The Forssman hapten may be extracted from tissues by alcohol; the purified hapten is soluble in water, dilute alkali, and pyridine, and is not soluble, or barely so, in most organic solvents. Reducing sugars are detected after hydrolysis, and the Forssman hapten must be mixed with proteins to produce antibodies.

On the other hand, the Boivin antigen is specific, toxic, and antigenic, and on hydrolysis fatty acids and a specific polysaccharide are liberated; the Brucella antigens are precipitated by alcohol, and are resistant to boiling; the *Brucella melitensis* antigen appears to be a complex of at least two phospholipids with protein-like material and the *N*-formyl derivative of a polyhydroxamine. The *Brucella abortus* antigen is probably similar chemically. The Forssman hapten is resistant to boiling, and the purified active moiety is

apparently not soluble, or barely so, in most organic solvents, including alcohol, ether, and chloroform (Landsteiner and Levene 1925); on hydrolysis, fatty acids, hexosamine, and probably a hexose are liberated (Boyd 1947).

Chemically, the serologically active lipid of *Haemonchus contortus* does not appear to belong to any of these types of lipoidal antigens; the common properties discussed are non-specific to each type and are probably shared by all compounds which contain a sufficiently high proportion of lipid.

The biological and available chemical evidence suggests that the serologically active lipids of the helminths studied by the author have a number of common features and are a type of compound not as yet found in serologically active preparations from other classes of organisms.

As regards the work of Fairley (1925, 1927), Kellaway (1928), and Wharton (1930) using bilharzia cercariae (*Schistosoma spindalis*), *Fasciola hepatica*, and *Moniezia expansa*, respectively, the type of material extracted in each case by alcohol (95 per cent. or higher) would be similar, although techniques varied slightly. Fairley and Wharton found that the alcoholic extracts of the helminths they studied could fix complement, and Kellaway suggested that the alcohol-soluble material of *Fasciola hepatica* was capable of complement fixation. For reviews of earlier work, which in the main support this hypothesis, reference should be made to these three authors.

All the serologically active lipids of helminths which have been studied have shown low specificity. Thus Wharton demonstrated the low specificity of tapeworm lipids and the present work shows that the absolute alcohol extracts of the nematodes and cestode studied (see Table 3) all fixed complement in the presence of *Haemonchus contortus* natural antisera from sheep with known histories, whereas alcoholic extracts of *Fasciola hepatica*, *Paramphistomum cervi*, and various vertebrate tissues did not. The fact that the absolute alcohol extract of *Moniezia expansa* fixed complement in the presence of *Haemonchus contortus* natural antisera in this Laboratory, implies that Wharton was dealing with a very similar, if not the same, lipid.

Since the serologically active lipid of *Haemonchus contortus* was found in greater amounts in the eggs and third-stage larvae and the active lipid content was not diminished in "aged" larvae, it is suggested that it functions as an essential lipid in the metabolism of *Haemonchus contortus*. Because of the similarity between the serologically active lipids of the helminths studied, it is possible that they are essential lipids involved in the basic metabolic structure of the helminths concerned.

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THE OCCURRENCE OF MUCOID SUBSTANCES IN INSECTS

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Summary

Three histochemical tests, which demonstrate mucoid substances of vertebrate origin, have been applied to a variety of insect tissues. Mucoid materials seem to be absent from the contents of the insect midgut, but a positive reaction may be given by the striated border of the epithelium. Goblet cells of the larval midgut of Lepidoptera and rectal glands of all of the insects studied give a negative reaction, but the salivary glands of the cockroach, grasshopper, larval calliphorids, and worker honeybee all contain mucoid substances. In general, these materials seem to be of less frequent occurrence in insects than they are in most other animal phyla. The significance of the observed distribution of mucoid substances in insects is discussed, particularly in relation to the functions of the peritrophic membrane and the salivary glands.

I. INTRODUCTION

There are many references to the occurrence of mucins in insects, but few of them are based on good evidence. Platania (1938) considered that the midgut of *Reticulitermes* produces a mucin which is incorporated in the lamellae of the peritrophic membrane, and refers to the statements, all published over 40 years ago, of six authors who reported "mucus" in the gut of a variety of insects. Ichikawa (1931) described a mucus layer in the gut of *Collembola*, and Weil (1936) also considered the peritrophic membrane of bees and wasps to have mucus incorporated in it. Hodge (1936) described a thin film of "mucous material" on the surface of the midgut epithelium of *Melanoplus*; the goblet cells in the midgut of larval Lepidoptera have been thought to secrete mucus (Frenzel 1886), and certain large cells of the *Psychoda* larval gut have been called mucous cells by Haseman (1910). But von Dehn (1933), Wigglesworth (1948), and others have maintained that mucins are absent from the insect gut. The current theory of the function of the peritrophic membrane in insects, namely that it serves to protect the midgut epithelium (Wigglesworth 1939), assumes that it replaces the mucins, which perform the protective function in many other groups of animals.

The rectal pads have been thought to secrete mucus (Sayce 1899; Marshall 1948); and the central cells of the cockroach salivary glands have been called mucous cells by Lebedeff (1899).

The contradictory conclusions in the majority of the above reports, and the uncertainty of the techniques employed (only mucicarmin and toluidine blue in most of the work) suggested the need to investigate the occurrence of mucoid substances in insects. The small quantity of material available from

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insect tissues precluded the detection of mucoid substances by any of the chemical tests that have been developed (see Burnet 1948), and so several histochemical methods, which give satisfactory results with vertebrate mucins, were employed.

Since the chemistry of none of the insect products has been studied it is not possible to classify them according to any modern scheme (for example, that of Meyer 1948), and for this reason the term "mucoid substance" has been used rather than the more specific mucin, mucopolysaccharide, mucoprotein, etc. The term mucoid substance will thus include all naturally occurring polysaccharides and protein-polysaccharide complexes in which at least part of the sugar moiety is a hexosamine.

II. METHODS

Tissues were fixed in Carnoy's, Helly's, and alcoholic Bouin's fluids. Carnoy's alcohol-acetic acid mixture was found to be the best of these and was used in all later work. Although aqueous fluids were avoided when possible, no improvement in the preparations resulted from spreading the paraffin sections on non-aqueous liquids. A number of mucin stains (mucihaematin, mucicarmine, thionine) were used, but later replaced by the following three histochemical tests:

- (1) The Gomori (1946) test for glycogen and mucin. Glycogen can be easily removed by ptyalin digestion and the test appears to be fairly specific; however, over-staining can result in a loss of specificity.
- (2) The Bismarck brown method (B) of Leach (1947), a technique resulting in a much improved specificity for water-labile mucoproteins.
- (3) The toluidine blue method, which gives a characteristic metachromasia with most mucoid substances, which Lison (1936) believes is practically a specific microchemical test.

Although none of these tests can be said to be absolutely specific, positive reactions with all of them provide a strong indication of the presence of mucoid substances.

III. OBSERVATIONS

An experiment was performed to determine which of the staining procedures, if any, gave the most easily detectable reaction with insect mucins. If any of the claims referred to above were justified it was to be expected that a positive reaction would be given by salivary glands, midgut goblet cells, and/or rectal pads. Sections of *Periplaneta* salivary glands and rectal pads and transverse sections of *Ephestia* larvae were fixed in Carnoy's, Helly's, and alcoholic Bouin's fluids and stained in toluidine blue, Gomori's methenamine, and Bismarck brown. The results are presented in Table 1.

From these data it is presumed that the *Periplaneta* salivary glands do include mucoid-containing cells, but that the rectal glands or goblet cells apparently do not. It was also clear that there were reactions in some other

tissues towards these histochemical tests. Thus the chitinous intima of the *Periplaneta* oesophagus, the cuticle of the *Ephestia* larvae, and the contents of the silk glands of *Ephestia* all gave positive results in some tests. Also from this experiment it appeared that the combination of Carnoy's fixative and Bismarck brown gave the best results. An attempt was then made to determine what tissues of a variety of insects gave this test for mucoid substances. Tissues showing a positive reaction were later checked by the other methods. Serial sections of several individuals of each of the following species were examined: *Ctenolepisma longicaudata* Esch., *Periplaneta americana* (L.), (embryos and adults), *Locusta migratoria* (L.), *Nasutitermes exitiosus* (Hill) (soldiers), *Coptotermes lacteus* (Froggatt) (soldiers), *Tenebrio molitor* L. (larvae and adults), *Ephestia kuhniella* Zeller (larvae), *Tineola biselliella* Hum. (larvae and adults), *Gnорimoschema operculella* Zeller (larvae), *Pieris rapae* (L.), (larvae), *Musca domestica* L. (larvae), *Lucilia cuprina* Wied. (larvae and adults), and *Apis mellifica* L. (workers).

TABLE 1
REACTIONS OF VARIOUS INSECT TISSUES TO TESTS FOR MUCOID SUBSTANCES

Tissue Test	<i>Periplaneta</i> Salivary Glands			<i>Periplaneta</i> Rectal Pads			<i>Ephestia</i> Larval Midgut Goblet Cells		
	Alc.			Alc.			Alc.		
	Carnoy	Helly	Bouin	Carnoy	Helly	Bouin	Carnoy	Helly	Bouin
Gomori's									
methenamine	+	±	+	—	—	—	—	—	—
Bismarck brown	+	±	+	—	—	—	—	—	—
Toluidine blue	±	+	±	—	—	—	—	—	—

+ indicates positive reaction; — indicates no characteristic stain observed; ± indicates probable positive reaction.

Of all these species positive reactions for mucoid substances were found in the following tissues.

- (1) Cuticle—for example, in *Ctenolepisma*, and in larvae of *Ephestia*, *Gnорimoschema*, *Lucilia*.
- (2) Chitinous intima—for example, in the foregut of *Periplaneta*, *Locusta*, *Lucilia*.
- (3) Striated border of midgut epithelium—*Ctenolepisma*; caeca and midgut of *Locusta*; midgut, but not crypts, of adult *Tenebrio*; midgut of *Pieris* and *Tineola*; and cells at the base of the crypts only, in *Apis*. This latter case was very specific and the reaction was clearly restricted to a few cells only in each crypt.
- (4) Striated border of only a few malpighian tubules of *Tenebrio*.

- (5) Fat body — some inclusions of larval fat body of *Lucilia* and the same tissue when found in recently emerged adults, and a diffuse reaction in the fat body of larvae of *Tenebrio* and of *Tineola*.
- (6) Peritrophic membrane — as in larvae of *Lucilia* and *Ephestia*, and in *Periplaneta*.
- (7) Connective tissue — as in the midgut of *Periplaneta*.
- (8) Salivary glands — *Periplaneta*, *Locusta*, *Apis*, larvae of *Lucilia* and *Musca*.

Of these examples a secretion of mucoid substances was observed only in the salivary glands. In the remainder the reaction was confined to formed cellular elements. In the cockroach salivary glands Lebedeff (1899) described acini containing two cell types. The peripheral cells were thought to produce digestive enzymes, whereas the central cells, which undergo a conspicuous cycle of secretory activity, were thought to produce mucin. The designations are not appropriate since "central cells" frequently occur on the periphery of the acini. But it is certainly these which produce the mucoid substance. However, not all the "central cells" give a positive reaction and it is evident that they do so only at certain periods of this secretory cycle. In *Periplaneta* starved for 14 days the "central cells" containing mucoid materials are greatly increased in number; in a *Periplaneta* fed only starch for 14 days the mucoid substance is very greatly depleted, and the cytoplasm of most of the central cells is markedly vacuolate. It is noteworthy that Day and Powning (1949) reported that the salivary glands of cockroaches similarly treated contained much less amylase than normal. Apparently mucin and amylase are secreted together. Preparations stained for polysaccharides by the method of McManus (1946) indicated that the cells giving the mucin reactions contained no stainable polysaccharide, although the "peripheral cells" were stained by this method.

In *Locusta* the "zymogenic cells" of Beams and King (1932) again give the positive reaction.

In *Apis* the "salivary glands" are well developed and are of several types (Kratky 1931). Only the cells of the lobes of pharyngeal glands give a positive reaction, and all cells appear to react with equal intensity.

In larvae of *Musca* and *Lucilia* the contents of the salivary glands give a strong positive reaction but the cells themselves do not, and in *Lucilia* at least this reaction is greatly enhanced in the prepupae at which time the glands become greatly swollen with a secretion of unknown function. The salivary glands of adult *Lucilia* gave a negative reaction. In the lepidopterous larvae examined the contents of the silk glands were weakly positive, but the cells of the glands were negative.

A positive reaction for mucoid substances was especially looked for in the goblet cells of the lepidopterous larval midgut in the region of peritrophic membrane formation in many species, in the head secretion of soldier termites, in

the reproductive tracts of all species studied, and in the embryonic tissues and imaginal buds, but the reaction was negative in all these examples.

It is likely that other tissues in other species might give positive reactions. For example, Glasgow (1936) describes dorsal cephalic glands of the larva of *Hydropsyche* and considers that they probably secrete mucus, but the above examples are sufficient to indicate the most usual sites of mucoid substances.

IV. DISCUSSION

(1) Before considering the distribution of positive reactions for mucoid substances in insect tissues it is appropriate to refer briefly to the types of materials included in this category. A recent review by Kurt Meyer (1948) includes three main types; the mucopolysaccharides, the mucoproteins, and the glycoproteins. Among the neutral mucopolysaccharides are examples giving on degradation residues of acetylglucosamine only—and chitins are typical of such materials. It is obvious, therefore, that the synthetic ability for mucopolysaccharide formation is highly developed in insects, and this, of course, explains the positive reaction of some cuticles to histochemical tests for mucoid substances. Also hyaluronic acid is an example of the acid mucopolysaccharides and, although it has not been proved, it is likely that this cementing substance occurs in insects as well as in vertebrates. Thus, cells of the midgut epithelium fall apart when soaked in a solution of hyaluronidase (Day and Powning 1949), and hyaluronidase itself has been extracted from a variety of insects (Duran-Reynolds 1939).

In view of the above it is all the more remarkable that other types of mucoid substances occur so infrequently in insects in comparison with their occurrence in vertebrates and some invertebrates (cf. Ewer and Hanson 1945; Kruidenier 1948).

(2) The absence of mucins in the lumen of the insect midgut (except their occasional presence in the peritrophic membrane) lends weight to the hypothesis that one of the principal functions of the latter is the protection of the midgut epithelium.

The fact that the striated border of some species gives a positive reaction (confirming the observation of Hodge 1936) does not weaken this argument; and it is interesting that Gersh (1948) found the striated borders of several vertebrate tissues also gave a positive reaction for glycoprotein.

(3) A similar parallel is not found in the goblet cells. In the vertebrate stomach and large intestine these are essentially producers of mucus. They must serve another function in the gut of lepidopterous larvae; none of the functions suggested for the goblet cells seem satisfactory.

(4) A comparison between the distribution of mucoid substances in vertebrates and insects is of interest. Dempsey and Wislocki (1946) and Wislocki *et al.* (1948) consider a number of locations of such substances in vertebrates. Of these, comparable sites occur in insects only in the stroma of

growing tissues, in some tissues which undergo repeated growth cycles (e.g. the midgut epithelium), in intracellular mucus, and in the secretion of certain glands. In the cockroach embryo, imaginal buds of larvae, and in the insect midgut no mucoid substances were found, and mucous glands appear to be much less frequent than in vertebrates. The salivary glands of insects are the only glands regularly found secreting mucoid materials.

(5) In vertebrates it has been reported that the sites of mucin formation also often give a reaction for alkaline phosphatase, and Leach (1947) has suggested that phosphatase may be a mucoprotein. No correlation between the locations of the two substances is found in insects (cf. Day 1949), indicating that none of the alkaline phosphatases found in insects are mucoproteins.

(6) The frequent occurrence of mucin in insect salivary glands suggests that it functions as a lubricant or to overcome harmful drying of the mouth-parts, which in the cockroach are moistened with salivary secretion during feeding (Wigglesworth 1939). This is substantiated by the absence of mucoid substances in some salivary glands whose function has been modified—as in lepidopterous larval silk glands. However, even in the silk gland of the webworm, *Hyphantria*, Kinney (1926) claims that a mucoid material surrounds the silk in the gland.

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THE HYDROGEN ION CONCENTRATION IN THE ALIMENTARY CANAL OF LARVAL AND ADULT LEPIDOPTERA

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Summary

The pH of the midgut digestive juices of two species of carnivorous lepidopterous larvae and of 40 species of adult Lepidoptera belonging to 16 families was determined. In all species it was alkaline. From these records, and from those available from the literature for phytophagous, wool-eating, and wax-eating lepidopterous larvae, it is possible to generalize that midgut alkalinity is characteristic of the order Lepidoptera and that it is not dependent upon feeding habits.

For those species for which it was determined, the blood was neutral or slightly alkaline, the crop contents had the same pH as that of the food offered, and the hindgut contents were slightly acid.

I. INTRODUCTION

The pH of the digestive juices has a very great influence on the activity of digestive enzymes and on the absorption of foodstuffs and poisons. Any generalizations that can be made for the midgut pH of insects, therefore, will help to provide a clearer picture of the extremely varied processes of digestion and absorption which occur in insects.

In most insects the midgut digestive juices vary only slightly from neutrality, generally falling within the pH range 6.0 to 8.0. Strong midgut acidity has seldom been recorded, but occurs in blowfly larvae, in blowfly adults, and in housefly larvae (Hobson 1931; Waterhouse 1940). It has also been reported for aphids (Bramstedt 1948) and for adult mosquitoes (MacGregor 1931), although the latter record is suspect in view of more recent studies (Fisk 1949; Popow and Golzowa 1933). At the other extreme, larvae of Lepidoptera (see Table 3) and Trichoptera (Shinoda 1930*b*) have weakly to strongly alkaline midgut contents, these generally varying from pH 8.0 to 10.0. In Coleoptera an alkaline reaction also occurs in the midgut of many foliage or wood-feeding species, whereas in many predacious species this region is weakly acid (Shinoda 1930*b*; Staudenmayer 1940). It is worthy of consideration, therefore, whether the occurrence of alkaline digestive juices in Lepidoptera is correlated with phytophagous habits or whether it is an attribute also of non-phytophagous forms and, therefore, a general characteristic of this order.

From records already available for lepidopterous larvae it is clear that distinct alkalinity is not limited to strictly phytophagous forms, since the midgut

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contents of larvae of the clothes moth (*Tineola biselliella*) have a pH of 9.9 and those of the wax moth (*Galleria mellonella*) have a pH of 8.4 (Duspiva 1935, 1936; Linderström-Lang and Duspiva 1936). However, it is known that both wool and wax are more readily degraded under alkaline than under acid conditions. Alkaline midgut juices would, therefore, favour digestion of these materials and may even have been responsible for permitting these species to adapt themselves successfully to their food. From available records, therefore, there is really no evidence upon which to decide whether alkalinity of the midgut contents in lepidopterous larvae is an adaptation to their food or whether it is a character of the order.

Two lines of approach to this problem were adopted. In the first, the pH of the digestive tract of two species of predacious lepidopterous larvae was investigated. Carnivorous insects frequently have neutral or acid midgut contents, and a pH above 8.0 would be regarded as unusual. In the second, the pH of the midgut of adult Lepidoptera was determined. If adults feed at all (and there are many species which do not) it is generally only on plant juices, such as nectar. There would not seem to be any advantage biochemically in having alkaline conditions for any of the enzymes required to split sugars, which alone can be utilized (Stober 1927). If, therefore, carnivorous larvae and nectar-feeding adults have alkaline midgut contents it may be concluded that midgut alkalinity is a characteristic of the order Lepidoptera. If, on the other hand, the digestive juices are not alkaline, this would be strong evidence that the midgut reaction was correlated with type of food.

The only available record of the pH of the midgut of adult Lepidoptera is that of Jameson and Atkins (1921), who reported a pH of 5.8 for the forepart of the midgut of the adult silkworm (*Bombyx mori*) when one gut was immersed in a solution of brom-cresol purple. Reasons for considering that this record is misleading are given later. Stober (1927) reported that the pupal residues still present in the midgut of several adult butterflies on emergence were acid. The indicators he used (which all changed at pH 7.0 or less) did not enable him to decide if, when the residues had been discharged, the digestive juices were neutral or weakly alkaline.

II. METHODS

Indicator paper and indicator feeding methods were used since the amounts of fluid available were often extremely small and since the latter method enables localized regions of different pH to be readily recognized. Although the use of indicators is subject to certain errors (Waterhouse 1940), their magnitude is not sufficiently great to alter any of the conclusions of this paper.

It was not found possible to induce the carnivorous larvae to ingest indicators. They were therefore carefully dissected in their own haemolymph and the alimentary canals transferred to indicator paper and blotted dry. The midgut was then punctured and the contents allowed to spread out into the

surrounding paper. This method appeared to be the only one feasible, at least for the smaller of the two species (*Stathmopoda melanochra*), which contained a large amount of dark red pigment derived from the bodies of the coccid on which it preys. Whereas the midgut fluid diffused out into the indicator paper the pigment was retained near the alimentary canal, thus permitting observation of indicator changes which would otherwise have been obscured by the pigment.

Adult Lepidoptera were starved for some hours and then allowed to feed on sugar solutions saturated with indicators. As a rule, when the tarsi were brought into contact with the sugar solution, an immediate uncoiling of the haustellum resulted and feeding followed. The adults were dissected at various intervals after feeding and the indicator coloration observed immediately through the walls of the alimentary canal. The indicators used in the feeding experiments all belonged to the sulphonphthalein series (thymol blue, cresol red, phenol red, brom-thymol blue, brom-cresol purple, chlor-phenol red, brom-cresol green, and brom-phenol blue). One member of this series, meta-cresol purple, gave aberrant colour changes in preliminary tests and was not used. All indicators were readily accepted except chlor-phenol red, which alone of the series has a definite phenolic smell.

The nomenclature of G. A. Waterhouse (1932) was followed for the butterflies examined, and the author is indebted to Mr. I. F. B. Common for identifying the moths.

III. RESULTS

(a) Larvae

The two species used were *Stathmopoda melanochra* Meyr. (Heliodinidae) the larvae of which prey upon the scale *Eriococcus coriaceus* Maskell, and *Titanoceros thermoptera* (Low.) (Pyralidae) the larvae of which eat the eggs of the Boree or bag shelter moth, *Ochrogaster contraria* (Walker).

As may be seen from Table 1, the larval midgut of both species is quite alkaline, pH 8.3 to 8.6 for *Stathmopoda* and 8.4 to 8.6 for *Titanoceros*.* The fluid regurgitated by *Stathmopoda* larvae, consisting of crop contents and saliva, had the same pH as the midgut contents. The blood of the two species was neutral or slightly alkaline.

(b) Adults

Forty species of adult Lepidoptera belonging to 16 families were examined and, in all species, the midgut contents were alkaline (Table 2). Both sexes gave similar results. The midgut pH of many species is shown in the Table as 8.4. In these species, the contents caused both phenol red and cresol red to exhibit their alkaline coloration, although thymol blue failed to do so, thus indicating a pH of about 8.4. There was some suggestion, however, from experiments in which the alimentary canal was immersed in buffers that thymol blue was not changing colour visibly at as low a pH as it should. It is possible,

* Since this paper went to press, *Catoblemma* larvae (Noctuidae) predacious on the scale *Cryptes* have been found to have alkaline (pH 8.2 to 9.6) midgut contents.

therefore, that the pH of the midgut contents in these species is actually higher than 8.4. The cabbage white butterfly (*Pieris rapae*) was the only species in which thymol blue gave some indication of its alkaline coloration. Here, the midgut contents are apparently slightly more alkaline than those of the other species tested.

TABLE 1
THE pH OF THE LARVAL MIDGUT AND BLOOD OF TWO SPECIES OF MOTH

Indicator Paper	<i>Stathmopoda</i>		<i>Titanoceros</i>	
	Midgut	Blood	Midgut	Blood
2-Cresol phthalein*			< 9.6	
Sodium 2,4-dinitrobenzol-azo-4'- naphthol-8-sulphonic acid*	< 8.8	< 8.8	8.2 to 8.8	
8410 wide range†	< 8.7	< 8.7	< 9.0	
Thymol blue	< 8.6	< 8.6	< 8.6	< 8.6
Universal†	8.0 to 9.0	7.0 to 7.5	c. 9.0	7.0 to 8.0
Disodium diamidostilbene- azophenol disulphonate*	c. 8.4	< 7.2	> 8.4	
6883 wide range†	> 8.3	< 7.4		c. 7.1
Cresol red	> 8.2	< 7.8	> 8.2	< 7.8
Disodium 2,4-dinitrobenzol-azo- 1-naphthol-3, 6-disulphonate*	> 8.0	6.5 to 7.5		c. 7.0
Phenol red	> 7.6	< 7.4		< 7.4
Brom-thymol blue	> 6.7	> 6.7		< 6.7
Range	8.3 to 8.6	c. 7.0	8.4 to 8.6	c. 7.0

* Bayer (Leverkusen) indicator papers.

† Johnson and Sons (London) indicator papers.

All except three of the butterflies examined had quite alkaline midguts. These three belonged to the family Satyridae and, although each possesses a well-developed haustellum, great difficulty was experienced in inducing them to take the sugar solutions. In fact, the majority of individuals starved to death after several days without feeding. In their natural habitat they are certainly not nectar-loving species; they are generally found in shady situations, and they are not associated with the presence of flowers. The slightly alkaline pH (7.6) of the midgut in these species may, therefore, be associated with their habit of feeding rarely, if at all.

The pH of the contents of the crop was always the same as that of the food offered, and the hindgut contents were acid, falling within the range

TABLE 2
MIDGUT AND HINDGUT pH IN ADULT LEPIDOPTERA

Species	Mid-gut	Hind-gut	Species	Mid-gut	Hind-gut
Hesperiidae			Lycaenidae (continued)		
<i>Toxidia peroni</i> (Latr.)	8.4		<i>Candalides acasta</i> Cox	8.4	6.4
<i>Padraona flavovittata</i> <i>flavovittata</i> (Latr.)	8.4	5.8-6.2	<i>Neolucia mathewi</i> Misk.)	8.4	6.4
<i>Taractocera papyria</i> <i>papyria</i> (Bois.)	8.4	5.8	Plutellidae		
Satyridae			<i>Plutella maculipennis</i> (Curtis)	7.6	6.0-6.5
<i>Heteronympha merope</i> <i>merope</i> (Fabr.)	8.4	6.0-6.9	Gelechiidae		
<i>Heteronympha philerope</i> (Bois.)	8.4		<i>Sitotroga cerealella</i> (Oe.)	7.6	5.8
<i>Oreixenica lathoniella</i> <i>herceus</i> Wat. and Lyell	7.6		<i>Gnorimoschema opercu-</i> <i>lella</i> (Zell.)	8.4	4.0-5.8
<i>Hypocysta pseudirius</i> Butl.	7.6		Oecophoridae		
<i>Hypocysta metirius</i> Butl.	7.6		<i>Endrosis lactella</i> (Schif- <i>fermüller)</i>	c. 7.5	
Nymphalidae			<i>Machimia carnea</i> (Zell.)	8.4	
<i>Precis villida calybe</i> God.	8.4	6.4	Tortricidae		
<i>Pyrameis cardui</i>			<i>Cydia pomonella</i> (L.)	8.4	
<i>kershawi</i> McCoy	8.4		<i>Tortrix postvittana</i> (Walk.)	8.4	
<i>Pyrameis itea</i> (Fabr.)	8.4		<i>Acropolitis dryinodes</i> Meyr.	c. 7.5	
Danaidae			Heliodinidae		
<i>Danaida plexippus</i> (L.)	8.4		<i>Stathmopoda melanochnra</i> Meyr.	7.6-7.8	
<i>Danaida chrysippus</i> <i>petilia</i> (Stoll.)	8.4		Zygaenidae		
Papilionidae			<i>Pollanisus apicalis</i> Walk.	8.4	
<i>Papilio sarpedon</i> <i>choredon</i> Feld.	8.4	6.4	Arctiidae		
<i>Papilio aegeus aegeus</i> Don.	8.4		<i>Thallarcha lochaga</i> (Meyr.)	c. 7.5	
Pieridae			<i>Asura lydia</i> (Don.)	c. 7.5	
<i>Pieris rapae</i> (L.)	8.4-9.0	6.4	Noctuidae		
<i>Terias smilax</i> (Don.)	8.4		<i>Cruria donovani</i> Bois.	c. 7.5	
<i>Delias nigrina</i> (Fabr.)	8.4		<i>Agrotis infusa</i> Bois.	c. 7.5	
<i>Delias harpalyce</i> (Don.)	8.4		<i>Heliothis armigera</i> (Fabr.)	8.4	
Lycaenidae			Sterrhidae		
<i>Zizeeria labridas</i> <i>labridas</i> (Cod.)	8.4	5.7-6.0	<i>Scopula rubraria</i> (Dbl.)	8.4	
<i>Lucia lucanus</i> (Fabr.)	8.4				
<i>Lampides boeticus</i> <i>damoetes</i> (Fabr.)	8.4				

5.7 to 6.9. When examining the hindgut it was observed that the contents were generally less acid at the anterior end than at the posterior end. This was particularly noticeable when indicator solution had recently passed on from the alkaline midgut. Similarly, indicator solution which had recently passed from the anterior into the middle and posterior segments of the hindgut was less acid than when it had been retained in these latter segments for some time. In several instances the excreta were slightly more acid than the hindgut contents, as in *Precis villida* (Nymphalidae), where the hindgut pH was generally about 6.4, whereas drops of excreta had a pH of about 5.8.

The rate of passage of food down the alimentary canal varied in different species, although in most it appeared to be fairly rapid and, in all, indicator solution filled the midgut within 30 minutes. The rate of passage was most rapid in starved individuals. In *Pieris rapae* food had already reached the middle segment of the hindgut in several instances 30 minutes after feeding commenced, whereas in *Precis villida* the corresponding time was about 60 minutes.

In 7 of the 16 species of moths the midgut pH was 8.4 or higher and in the remaining species it was about 7.5, the turning point of phenol red. In some of these species (e.g. *Stathmopoda melanochra*) the midgut often appeared to be slightly more alkaline at the posterior than at the anterior end, although in others (e.g. *Gnorimoschema operculella*) the reverse was true.

In most moths, and particularly those with a midgut pH about 7.5, the digestive tract appears to be very poorly buffered. Thus, food passed recently from the crop to the midgut does not change rapidly to the characteristic alkaline pH of this region and, if adults are examined within an hour or two after feeding, the pH of the midgut is seen to be variable. However, several hours after feeding, the midgut contents have generally become alkaline, although at any time they may be altered by further passage of food from the crop. Similarly, the pH of the hindgut contents of moths is more variable than that of the butterflies examined, the rectal contents of the potato moth *Gnorimoschema*, for example, varying in different individuals from about 4.0 to about 5.8. In dissected individuals the acidity of the hindgut contents can be observed to diminish rapidly with the passage of alkaline fluid from the midgut. In recording results in Table 2, it has been assumed that the highest alkalinity observed in the midgut and the greatest acidity in the hindgut are typical of the secretions of these regions.

IV. DISCUSSION

It is clear from these results and from the published records summarized in Table 3, that the alkalinity characteristic of the midgut contents of larval Lepidoptera cannot be regarded simply as an adaptation to phytophagous habits, for alkalinity is characteristic also of carnivorous larvae and of nectar-feeding adults. The degree of alkalinity varies within a rather wide range, from species

TABLE 3
RECORDS OF MIDGUT AND HINDGUT pH IN LEPIDOPTERA
(FIGURES GIVEN TO NEAREST 0.1 pH UNIT)

Species	Stage	Midgut	Hindgut	Author
Nymphalidae				
<i>Vanessa urticae</i>	Larva	8.7		Staudenmayer and Stellwaag 1940
Pieridae				
<i>Pieris brassicae</i>	"	9.4		Skrjabina 1936
" "	"	8.0		Staudenmayer and Stellwaag 1940
<i>P. napi</i>	"	9.3		Skrjabina 1936
Tineidae				
<i>Tineola biselliella</i>	"	9.9		Duspiva 1936
" "	"	9.6-10.2		Linderström-Lang and Duspiva 1936
Hyponomeutidae				
<i>Hyponomeuta malinella</i>	"	9.2		Skrjabina 1936
Tortricidae				
<i>Cydia molesta</i>	"	7.2-7.3	7.2	H. S. Swingle 1928
<i>C. pomonella</i>	"	8.5-8.7	7.8	Marshall 1936
<i>Polychrosis botrana</i>	"	7.7		Staudenmayer and Stellwaag 1940
<i>Clysia ambiguella</i>	"	7.6		Staudenmayer and Stellwaag 1940
Galleriidae				
<i>Galleria mellonella</i>	"	8.0-8.4		Duspiva 1935
" "	"	8.4		Duspiva 1936
" "	"	7.0-8.0	6.4-6.6	Roy 1937
Notodontidae				
<i>Datana integerrina</i>	"	9.5		H. S. Swingle 1938
Noctuidae				
<i>Heliothis obsoleta</i>	"	8.0		M. C. Swingle 1931
" "	"	8.0		H. S. Swingle 1938
<i>Panolis flammea</i>	"	7.6	7.3	Trappmann and Nitsche 1933
<i>Barathra brassicae</i>	"	9.6		Skrjabina 1936
<i>Euxoa segetum</i>	"	9.7		Skrjabina 1936
Lymantriidae				
<i>Lymantria dispar</i>	"	8.5	8.2	Trappmann and Nitsche 1933
" "	"	9.4		Skrjabina 1936
<i>L. monacha</i>	"	8.3	7.3	Trappmann and Nitsche 1933
<i>Euproctis chrysorrhoea</i>	"	9.6		Skrjabina 1936

TABLE 3 (continued)

Species	Stage	Midgut	Hindgut	Author
Geometridae				
<i>Cheimatobia brumata</i>	Larva	9.5		Skrjabina 1936
Sphingidae				
<i>Protoparce sexta</i>	"	9.6		H. S. Swingle 1938
<i>Ceratonia catalpa</i>	"	9.5	5.7	M. C. Swingle 1931
<i>Deilephila euphoribae</i>	"	> 7.5	< 5.0	Stober 1927
" "	"		5.8-6.3	Heller and Aremowna 1932
Lasiocampidae				
<i>Malucosoma neustria</i>	"	9.3		Skrjabina 1936
Bombycidae				
<i>Bombyx mori</i>	Adult	5.8	5.2	Jameson and Atkins 1921
" "	Larva	9.0-9.8	8.4	Jameson and Atkins 1921
" "	"	8.0, fed 9.6, starved		Suzuki 1924
" "	"	10.3		Gamo 1928
" "	"	10.0		Gamo <i>et al.</i> 1933
" "	"	9.8		Shinoda 1930
" "	"	9.1	8.9	Trappmann and Nitsche 1933
" "	"	9.8		Skrjabina 1936
" "	"	9.2 to 9.8, fed 10.0, starved		Itaya 1936

in which the contents are only slightly alkaline to those in which a pH of 10.0 or higher has been recorded, e.g. 10.3 for *Bombyx mori* larvae (Gamo 1928).

Although it is possible that the midgut of some lepidopterous larvae and adults yet to be investigated may be found to be neutral or acid, the fact remains that, of 26 species of larvae and 41 species of adults for which records are available (Tables 1, 2, and 3), there is only one record of midgut acidity and this is for the silkworm adult (Jameson and Atkins 1921). This record may be questioned on a number of grounds. It is based on one individual only; the method used of breaking the gut in a drop of indicator is not a particularly reliable one since the pH may be influenced by that of the cell contents; and, most important of all, adult *B. mori* do not take food and, therefore, the record is not for functional digestive juices. Furthermore, the midgut is said to be the site of secretion of an alkaline fluid which the emerging adult uses to soften the cocoon (Itaya 1936). One may conclude, therefore, that the record of Jameson and Atkins (1921), even if valid, is not an exception to the generalization that the midgut digestive juices in both larval and adult Lepidoptera are alkaline irrespective of their food.

No generalization can be made about the reaction of the hindgut except that it is never as alkaline as the midgut. In some species it is quite alkaline (e.g. pH 8.4 to 8.9 in *B. mori* larvae), although in most it is mildly acid.

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THE EFFECT OF SYMPATHECTOMY ON WOOL GROWTH

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Summary

Five sheep (two Merinos, two Corriedales, and one crossbred) were subjected to unilateral thoracic sympathectomy. Fleece samples were collected at 28-day intervals, for a period of seven months, from tattooed areas of skin on both the sympathectomized and control sides.

The results show a mean increase of 36 per cent. in wool growth rate on the sympathectomized side over the control side for ten weeks after the operation. This effect then disappeared so that no difference between the two sides was observed over the remainder of the experimental period. It is suggested that the initial effect of sympathectomy on wool growth rate was brought about by vasodilation of the denervated vessels and that the subsequent disappearance of this effect was due to the onset of warmer weather causing vasodilation of the control side.

Skin surface temperature showed a significant increase on the sympathectomized side immediately after the operation but this effect also disappeared within a few weeks so that no difference was detected during the fleece collection periods. Skin surface temperature may have been depressed on the sympathectomized side during the process of actual measurement due to constriction of the denervated cutaneous vessels caused by nervous excitement.

I. INTRODUCTION

Recent observations on the relation of wool growth rate to atmospheric temperature (Ferguson, Carter, and Hardy 1948) might be explained by the well-known effect of environmental temperature on the peripheral blood circulation with concomitant variation of the nutrient supply to the wool follicles. As such an effect, if present, is probably mediated via the sympathetic vasoconstrictor nerves to the cutaneous vessels, it follows that sympathectomy would increase wool growth and thus tend to confirm our explanation. This hypothesis is supported by the observation that section of the sympathetic nerves to one ear in rabbits and cats causes a speedier regrowth of hair on the denervated side (Pye-Smith 1887; Grant 1935) and has now been subjected to further experimental test in the sheep.

II. MATERIAL AND METHODS

The experimental sheep were all ewes and comprised two Corriedales, two Merinos, and one crossbred. Two sheep were sympathectomized on the right side and three on the left. Under barbiturate anaesthesia, an incision about

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10 inches long was made parallel to the 9th rib. The periosteum was stripped from the 9th and 10th ribs and about 6 inches of bone from the dorsal end of each removed. Without penetrating the pleura, the sympathetic trunk was reached in the dorsal part of the chest cavity. Five ganglia (8th-12th thoracic) and the intervening trunk were removed.

Fleece growth was measured by clipping areas of 100 sq. cm. delineated by a tattooed square on each midside region. Measurement was commenced from 2 to 6 weeks after the operations on 4 of the sheep: clippings were taken at fortnightly intervals for 4 weeks and subsequently at 4-weekly intervals. The 5th sheep (F 338) was added to the experiment 4 weeks after the others and 16 days after she had been sympathectomized. Fleece samples for the determination of fibre length and fibre diameter were taken from the anterior margin of each tattooed area.

Prior to the first fleece-collection period, the sheep were closely clipped over the whole surface of the body and three of them were clipped again later in the experiment to note whether the removal of the fleece insulation affected the sympathectomized and control sides differently, as it might be expected to do.

One of the sheep (UNI. 2) lambed shortly after being sympathectomized. The lamb was removed 4 weeks after the commencement of the first collection period. This sheep died from an abscess on the shoulder 8 weeks later.

No attempt was made to control the plane of nutrition strictly from period to period in that the diet was prepared as required and not mixed beforehand for the whole experiment. Variations in dietary intake were, however, unimportant, as the experimental comparisons were between the sides of each sheep and not between sheep. Two sheep (254 and 249) were given 450 g. of feed per day, later increased to 900 g. per day. The other three (UNI. 2, F 338, and N.T.) were given 600 g. per day throughout except for UNI. 2 which was fed *ad libitum* on lucerne chaff while nursing her lamb.

Measurements of the temperature of the skin surface at 9 points on each tattooed area were made after each fleece clipping by means of a nichrome-constantan thermocouple connected to a potentiometer.

III. RESULTS

Examination of the data showed an increase in wool growth on the sympathectomized over the control side except in one sheep (UNI. 2). However, after 12 weeks of measurement there was a rather sudden disappearance of the difference between the two sides in all except one sheep (249). The data are, therefore, conveniently summarized by taking the mean values for each sheep over the first 12 weeks of measurement and over the remainder of the experiment (Table 1). The values of wool, wax, and suint weight are given per tattooed area per 28 days.

In the Table, the mean increase of values on the sympathectomized side as a percentage of the control side is shown, and the probabilities that the

mean differences between the sides would be exceeded for reasons other than the operative treatment are also indicated. "Student's" *t* distribution was used to calculate the probability values. In the first period the probabilities for wool weight, fibre length, and possibly fibre diameter, are suggestive of a real treatment effect. Disappearance of the effect in the second period strengthens the conclusion of a true effect in the first, as the largest source of error variation is likely to have come from pre-treatment differences between the sides which would not tend to disappear.

As the samples for measurement of fibre length and diameter were not from exactly the same area as those for that of wool weight, it cannot be expected that the observed changes in length and diameter will account exactly for the changes in wool weight, assuming that sympathectomy does not affect the number of fibres growing. However, with this assumption, the observed increases in fibre length and diameter give a theoretical increase in wool weight of 32 per cent. for the first period which corresponds reasonably well with the observed value of 36 per cent.

The Table does not indicate a significant effect of the operation on the skin temperature of the tattooed area. In fact, the temperature on the control sides has a tendency to be higher. However, in the first measurement of skin temperature at the beginning of the first fleece-collection period, the mean temperature was slightly higher (0.8°C.) on the sympathectomized side, and earlier measurements on one sheep (UNI. 2) showed a temperature increase of several degrees on the side operated upon which diminished as the time after the operation increased. In only one sheep (249) did the temperature remain greater on the sympathectomized side throughout the experiment. This was also the only sheep in which a difference in wool growth rate persisted.

Although individual differences in wax weight between "operated" and control sides are rather large they are not consistent in direction and cannot be ascribed to the treatment. It must be concluded either that the treatment has affected wax production differently in the several individuals or that the differences existed prior to the treatment. The differences cannot be due to random variation in technique of wax extraction as the 4-weekly analyses consistently maintain the mean differences shown in the Table. One might infer from the data that sympathectomy reduces suint secretion but the probability values are too high to allow this conclusion to be drawn.

No consistent effect of clipping the sheep closely all over on the difference in fleece growth between the sides was observed.

IV. DISCUSSION

The rapid disappearance of the skin temperature difference between the sides is in accord with the observed recovery of vascular tone following denervation first reported by Goltz and Freusberg (1874) and later repeatedly

TABLE 1
RESULTS OF SYMPATHECTOMY ON FLEECE GROWTH, GLAND SECRETIONS, AND SKIN SURFACE TEMPERATURE

RESULTS OF SYMPATHECTOMY ON FLEECE GROWTH, GLAND SECTIONS, AND														
Sheep No.	Date of Operation	Side	June 29 to September 8, 1948						September 9, 1948 to January 26, 1949					
			Wool Weight (g.)	Fibre Length (mm.)	Fibre Diameter	Wax Weight (g.)	Suint Weight (g.)	Skin Temperature (°C.)	Wool Weight (g.)	Fibre Length (mm.)	Fibre Diameter	Wax Weight (g.)	Suint Weight (g.)	Skin Temperature (°C.)
249 (Corriedale)	27.v.1948	S* (left)	1.193	11.62	26.8	0.387	0.089	32.00	2.226	11.52	27.4	0.365	0.175	33.50
		C** (right)	0.799	9.26	24.0	0.237	0.090	31.69	1.943	10.83	25.1	0.299	0.187	32.98
		Difference	0.394	2.36	2.8	0.150	-0.001	0.31	0.283	0.69	2.3	0.066	-0.012	0.52
254 (Corriedale)	2.vi.1948	S (right)	0.622	10.55	25.4	0.433	0.086	31.30	1.522	11.53	27.3	0.370	0.108	33.11
		C (left)	0.348	8.52	21.7	0.218	0.053	31.22	1.682	11.13	26.2	0.342	0.144	34.00
		Difference	0.274	2.03	3.7	0.215	0.033	0.08	-0.160	0.40	1.1	0.028	-0.036	-0.89
UNL. 2 (crossbred)	6.v.1948	S (right)	0.943	9.10	24.1	0.360	0.073	32.76	Dead					
		C (left)	0.941	9.41	22.4	0.510	0.091	33.31						
		Difference	0.002	-0.31	1.7	-0.150	-0.018	-0.55						
N.T. (Merino)	17.v.1948	S (left)	1.474	8.60	24.2	1.230	0.195	32.40	1.179	8.04	21.5	0.595	0.207	35.05
		C (right)	1.309	7.93	26.0	1.475	0.293	32.54	1.114	7.84	22.5	0.659	0.199	35.19
		Difference	0.165	0.67	-1.8	-0.245	-0.098	-0.14	0.065	0.20	-1.0	-0.064	0.008	-0.14
F 338 (Merino)	28.vi.1948	S (left)	1.766	9.54	22.6	0.771	0.092	31.20	1.092	9.36	21.5	0.584	0.305	34.70
		C (right)	1.002	7.86	20.4	1.052	0.249	31.77	1.220	9.29	21.2	0.667	0.411	34.81
		Difference	0.764	1.68	2.2	-0.281	-0.157	-0.57	-0.128	0.07	0.3	-0.083	-0.106	-0.11
Mean		S	1.200	9.88	24.6	0.636	0.107	31.93	1.505	10.11	24.4	0.478	0.199	34.09
		C	0.880	8.60	22.9	0.698	0.155	32.11	1.490	9.77	23.8	0.494	0.235	34.24
		Difference	0.320	1.29	1.7	-0.062	-0.048	-0.17	0.015	0.34	0.7	-0.016	-0.036	-0.15
% Increase on Sympathectomized Side			36	15	7	-9	-31	-0.6	1	3	3	-3	-15	-0.3
		Probability	0.07	0.06	0.14	0.57	0.24	0.37	0.89	0.09	0.94	0.68	0.24	0.64

* Sympathectomized. ** Control.

confirmed. Grant (*loc. cit.*) in a detailed investigation of the effect of denervation of the rabbit's ear found that vascular tone returns in 5-7 days. Other investigators have found similar periods of recovery.

The return of vascular tone is accompanied by a greater sensitivity of the vessels to vasoconstrictor and vasodilator substances and to local mechanical and thermal stimuli (Elliott 1905; Dale and Richards 1918); Freeman, Smithwick, and White 1934; Grant *loc. cit.*). It seems possible that the recovery of vascular tone is due to the response of the more reactive denervated vessels to a circulating vasoconstrictor substance. Grant's work suggests that the substance is liberated during nervous excitement or muscular activity and that when the animal is completely relaxed the denervated vessels become dilated no matter whether the normal vessels are constricted or dilated. Of importance to the present discussion is the fact that increased heat loss by general cooling of the body does not cause constriction of the denervated as it does of the normal vessels as long as the animal remains relaxed. The nature of the hypothetical vasoconstrictor substance and the explanation of the greater reactivity of denervated vessels are at present unknown.

The discrepancy in the differences in wool growth rate and skin temperature between sides of the sheep may therefore be due to insufficient relaxation of the sheep at the actual time of measurement of skin temperature so that the values for the denervated side were below the mean value for the wool growth period. A continuous record of skin temperature may have shown differences in skin temperature more comparable with the wool growth values.

The disappearance of the difference in wool growth rate between sympathetomized and control sides occurred at about the same time in the sheep operated on at different periods and coincided with the onset of warmer weather. It is suggested that the warmer conditions reducing heat loss to a critical point caused a reduction of vasoconstrictor impulses to the normally innervated vessels thereby increasing blood flow and wool growth rate to the same level as the sympathetomized side. Such an explanation would be substantiated by a recurrence of the difference between the sides with the return of colder weather.

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